Biosynthesis and Assembly of Very Long Chain Polyunsaturated Fatty Acids in *Thraustochytrium* sp. 26185

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By

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ABSTRACT

Thraustochytrium is a marine protist producing a specific profile of nutritionally important fatty acids, including very long chain polyunsaturated fatty acids (VLCPUFAs) docosahexaenoic acid (DHA, 22:6n-3), even-chain saturated fatty acids (SFAs) palmitic acid (16:0), and odd-chain SFAs pentadecanoic acid (15:0). However, the mechanism of how these fatty acids are synthesized and assembled into the storage lipid triacylglycerol (TAG) is unclear.

Firstly, we reported sequencing of the whole genome and genomic analysis of genes involved in the biosynthesis and assembly of the fatty acids in this species. Analysis of annotated genes from the genome revealed co-existence of both aerobic pathway and anaerobic pathways for the biosynthesis of VLCPUFAs in this species. However, in the aerobic pathway, a key gene encoding stearoyl Δ9 desaturase introducing the first double bond to long chain saturated fatty acid 18:0 was missing from the genome. A genomic survey of genes involved in the acyl trafficking among glycerolipids showed that, unlike plants, this protist did not possess phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), an important enzyme in bridging two types of glycerolipids, diacylglycerol (DAG) and phosphatidylcholine (PC).

Secondly, a series of radiolabeled precursors were used to trace the biosynthetic process of different fatty acids *in vivo* and *in vitro*. When *Thraustochytrium* was fed with long chain fatty acid intermediates such as ^{14}C-oleic acid, ^{14}C-linoleic acid and ^{14}C-α-linolenic acid, no VLCPUFAs were produced, indicating that the aerobic pathway for the biosynthesis of VLCPUFAs was not functional in *Thraustochytrium*. When fed with ^{14}C-acetic acid, both SFAs and VLCPUFAs were labeled, and when fed with ^{14}C-propionic acid, mainly SFAs were labeled. However, when fed with ^{14}C-acetic acid in the presence of cerulenin, a type I FAS inhibitor, only VLCPUFAs were labeled, and when fed with ^{14}C-propionic acid in the presence of cerulenin, neither SFAs nor VLCPUFAs were labeled. This result clearly indicates that the type I fatty acid synthase (FAS) in *Thraustochytrium* could use acetic acid and propionic acid as the primers to synthesize even-chain and odd-chain SFAs, respectively, and VLCPUFAs were synthesized by the PUFA synthase using acetic acid as the primer. The *in vitro* assay with ^{14}C-malonyl-CoA in the presence of cerulenin showed that the crude protein of *Thraustochytrium* produced only VLCPUFAs, not SFAs, further confirming the role of the PUFA synthase in the biosynthesis of VLCPUFAs. These results have elucidated the biochemical mechanisms for the biosynthesis of all fatty acids in *Thraustochytrium*.

Thirdly, how freshly-synthesized fatty acids are incorporated into different glycerolipids was investigated. The glycerolipid profile of *Thraustochytrium* at log and stationary growth stages was analyzed by lipidomic tools, and then ^{14}C-acetate and ^{14}C-glycerol were used to trace the flux of fatty acids and backbone in glycerolipids. Lipidomic analysis showed that VLCPUFAs were mostly allocated to phosphatidylcholine (PC) and TAG. PC possessed a relatively stable profile of VLCPUFAs at the two growth stages, whereas TAG species with one or two VLCPUFAs were significantly increased at the stationary phase. Freshly-synthesized VLCPUFAs labeled by ^{14}C-acetate were predominately incorporated into PC initially, while at the late time point of labeling, these fatty acids were mostly found in TAG. Positional analysis showed that PC had either one VLCPUFA at its *sn*-2 position (PC1) or two VLCPUFAs at both *sn*-1 and *sn*-2 positions (PC2), while TAG incorporated these fatty acids almost exclusively at the *sn*-2 position with similar stereospecific structure as PC1. Similarly, ^{14}C-glycerol was more efficiently incorporated into PC1 than TAG initially, and at the late time point of labeling, it was mostly found in TAG, and DAG and PC1 shared a similar incorporation pattern. These results indicate that VLCPUFAs in TAG are mainly channeled from PC likely through diacylglycerol as the intermediate.

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I would like to give great acknowledgements to my supervisor Dr. Xiao Qiu for his wholehearted support. My research would not have been able to be completed without his continuous guidance and encouragements. As a student with a food science background working on a project more related with biochemistry, I encountered great challenges in getting myself onto the right track. It was Dr. Qiu who has always been there to help me out. I would also like to thank my committee members Drs. Takuji Tanaka, Darren Korber, Vladimir Vajanovic, Patrick Covello and my external examiner Owen Rowland for their help during my study. I would also like to thank my lab mates for every kind help they offered to make my four years of Ph.D. study a very memorable one. Their names are Dr. Dauenpen Meesapyodsuk, Dr. Cunmin Qu, Yan Chen, Jin Wang, Indika Beneragam, Xi Xie, Carla Almendariz, Shengjian Ye, Yi Chen and Kira Wang.

This dissertation is dedicated to my parents for their greatest support for my education since the time I was a little pupil. While most of my classmates did not make it to high school because of funding, my parents worked like a horse to make it easy for me all the way to university. Especially, I thank destiny for bringing my wife Liying Li into my life while I was at the University of Saskatchewan. I cannot image what my life would be without her. My heart settles when I know I am going to spend the rest journey of my life with her. I believe life will be better and better when we hold on to each other in difficulties.

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List of abbreviations

ACC	acyl-CoA carboxylase
ACP	acyl carrier protein
ACS	acyl-CoA synthetase
ALA	α-linolenic acid
ARA	arachidonic acid
AT	acyl transferase
BLAST	basic local alignment search tool
CDS	CDP-DAG synthase
CDP	cytosine diphosphate
CK	choline kinase
CL	cardiolipin
CMP	cytosine monophosphate
CPT	choline phosphotransferase
CTP	cytosine triphosphate
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DH	dehydrase
DHA	docosahexaenoic acid
DHAP	dihydroxyacetone-phosphate
DHAP-AT	dihydroxyacetone-phosphate acyltransferase
DPA	docosapentaenoic acid
DPM	disintegration per minute
EK	ethanolamine kinase
EPA	eicosapentaenoic acid
EPT	ethanolamine phosphotransferase
ER	enoyl reductase
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FFA	free fatty acid
G3P	glycerol-3-phosphate

GC	gas chromatography
GLA	γ-linolenic acid
GO	gene ontology
GPAT	glycerol-3-phosphate acyltransferase
GPC	glycerol-3-phosphocholine
GPCAT	glycerol-3-phosphocholine acyltransferase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOG	EuKaryotic Orthologous Groups
KR	ketoacyl reductase
KS	ketoacyl synthase
LA	linoleic acid
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
LPAT	lyso-phospholipid acyltransferase
LPC	lyso-phosphatidylcholine
LPCAT	lyso-phosphatidylcholine acyltransferase
LPE	lyso-phosphatidylethanolamine
MAG	monoacylglycerol
MAT	malonyl-CoA ACP transacylase
MBOAT	membrane-bound O-acyltransferase
MGAT	monoacylglycerol acyltransferase
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PCCT	phosphocholine cytidylyltransferase
PDCT	phosphatidylcholine: diacylglycerol cholinephosphotransferase
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PKS	polyketide synthase

PL	phospholipid
PLA2	phospholipase A2
PPTase	phosphopantetheinyl transferase
PS	phosphatidylserine
PSD	phosphatidylserine decarboxylase
PSS	phosphatidylserine synthase
PUFA	polyunsaturated fatty acid
SDA	stearidonic acid
SFA	saturated fatty acid
TAG	triacylglycerol
TLC	thin layer chromatography
VLCPUFA	very long chain polyunsaturated fatty acid
WS	wax synthase

1. Introduction

1.1 Rationale

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential for human health. Dietary supplementation of VLCPUFAs has shown that these fatty acids can improve the performance of eyes, brain and immune systems, as well as provide protection against various chronic diseases. *Thraustochytrium* sp. 26185 is a marine protist that can produce a high level of VLCPUFAs especially DHA in neutral lipid triacylglycerols (TAG). This source of VLCPUFAs has been used for functional food and animal feeds. However, synthesis and assembly of VLCPUFAs in this species is not well understood. Particularly, it remains elusive how VLCPUFAs, after being synthesized, are trafficked among glycerolipids and finally accumulated in the storage lipid. The objective of this project is thus to analyze the biosynthesis of fatty acids and probe the acyl flux using radioisotope tracers to elucidate the biosynthetic process and assembly pathway of VLCPUFAs in *Thraustochytrium* sp. 26185. The knowledge gained from this research will contribute to a better understanding of the accumulation of VLCPUFAs in the native microbial species and assist in formulating strategies for the heterologous production of VLCPUFAs in other systems.

1.2 Hypothesis

Very long polyunsaturated fatty acids are synthesized from the initial precursor acetic acid and are assembled into two major types of glycerolipids phosphatidylcholine and triacylglycerols in *Thraustochytrium*.

1.3 Objectives

The overall objective of this study is to elucidate the biosynthetic mechanisms and the assembly pathways of VLCPUFAs in *Thraustochytrium* sp. 26185. The specific objectives are as follows.

1. Sequencing and analysis of *Thraustochytrium* sp. 26185 genome to identify genes involved in the biosynthesis and assembly of fatty acids in *Thraustochytrium* sp. 26185.

2. Analysis of the biosynthetic process of fatty acids in *Thraustochytrium* sp. 26185 using a series of radioactive precursors.
3. Analysis of acyl flux among glycerolipids using ^{14}C-acetate and ^{14}C-glycerol tracers.

2. Literature Review

2.1 Very long chain polyunsaturated fatty acids (VLCPUFAs): definition, sources and health benefits

The definition of very long chain polyunsaturated fatty acids (VLCPUFAs) differs among researchers in terms of the chain length [1-3]. In this thesis, VLCPUFAs are defined as fatty acids with a chain length equal to or greater than 20 carbon atoms and with two or more carbon-carbon double bonds. According to the position of the first double bond from the methyl end, they are classified into two families, ω-3 (n-3) and ω-6 (n-6) VLCPUFAs. The nutritionally-important ω-3 VLCPUFAs mainly include eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), while the ω-6 VLCPUFAs mainly include dihomo-γ-linoleic acid (DGLA, 20:3n-6) and arachidonic acid (ARA, 20:4n-6). The double bonds of naturally occurring VLCPUFAs are mostly in a *cis* configuration, making these fatty acids a structure of multiple 'kinks' (Figure 2-1). These kinks in the phospholipid tails of cell membrane help to maintain membrane fluidity at low temperatures by 'elbowing' adjacent phospholipid molecules away, maintaining space between the phospholipid molecules.

Oils rich in these fatty acids occur naturally in the body lipids of fatty fish, the liver of white lean fish, the blubber of marine mammals, marine algae, fungi and bacteria. However, except some marine microorganisms, most bacteria, plants and animals cannot synthesize the VLCPUFAs *de novo*. Some marine bacteria such as *Moritella marina*, *Shewanella oneidensis* and *Colwellia psychrerythraea* can produce EPA and DHA under anaerobic conditions [4]. Some marine fungi, microalgae and protozoa are also able to synthesize VLCPUFAs [5]. Among them, *Thraustochytrium* and *Schizochytrium* are highly valued because they can accumulate, in their storage lipids, high concentrations of ω-3 VLCPUFAs, especially EPA and DHA [6]. Through the food chain, these fatty acids end up in marine fish and eventually in human diet and animal feeds. As fish oil is the main dietary source of VLCPUFAs for humans, this source has been overexploited. Due to overfishing and ocean pollution, the source is currently under intense pressure [7]. In addition, this source of VLCPUFAs contains contaminants including heavy metals, antibiotics, and carcinogens detrimental to human beings, especially children, pregnant and lactating women [8]. Besides the sustainability issue, fish oil is also undesirable in odor and is expensive to extract from fish.

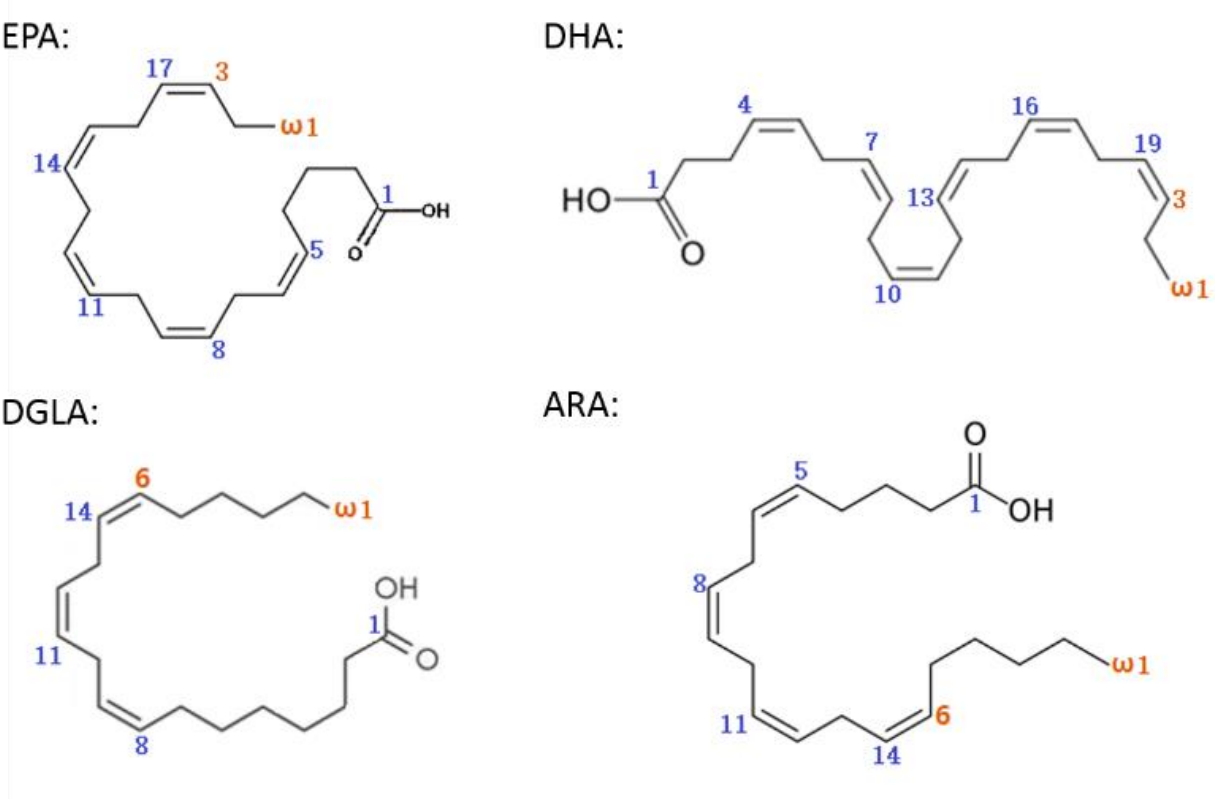

Figure 2-1. Chemical structures of EPA, DHA, DGLA and ARA.

Oilseed crops like canola, soybean, peanut and sesame can produce a large amount of 18-carbon unsaturated fatty acids including linoleic acid (LA) and α-linolenic acid (ALA). When microbial desaturases and elongases are introduced into these plants, they are able to produce VLCPUFAs from endogenous long chain PUFAs. Some transgenic lines of oilseed crops carrying microbial VLCPUFA biosynthetic pathways such as *Camelina sativa* [9, 10], *Brassica juncea* [11] and soy bean *Glycine max* [12] produce high levels of VLCPUFAs, which can serve as alternative sources of fish oil. In addition, metabolic engineering of VLCPUFA-producing microalgae and fungi can dramatically enhance their fatty acid accumulation, making them possible for commercial production of such fatty acids. For example, the DHA content in microalga *Phaeodactylum tricornutum* was increased by eight-fold when exotic desaturases and elongases were expressed [13]. When an ω-3 desaturase was introduced to the ARA-producing oleaginous fungus *Mortierella alpina*, the content of EPA was increased dramatically [14]. When ribulose 1,5-bisphosphate carboxylase/oxygenase was disrupted in the DHA-producing microalga, *Crypthecodinium cohnii*, to upregulate energy metabolism and direct more carbon for lipid accumulation, the DHA production was observed to increase significantly [15]. Such metabolic strategies make oilseed crops, marine fungi and microalgae alternative sources of VLCPUFAs to fish oil.

The benefits of ω-3 and ω-6 VLCPUFAs to human health have been extensively investigated and many clinical data are available to advocate the functions of these fatty acids. However, evidence disproving the health benefits of these fatty acids, especially ω-6 VLCPUFAs, have also been accumulating in recent years. VLCPUFAs are the precursors of eicosanoids and docosanoids such as prostaglandins, thromboxane, resolvins, docosatrienes and neuroprotectins. These compounds play important roles in immunity, blood clots, neurotransmitters, cholesterol metabolism and structure of membrane phospholipids in brain and retina [5]. It is becoming certain that ω-6 eicosanoids and ω-3 docosanoids/eicosanoids have almost diametrically-opposing physiological and pathological activities [16, 17]. Researchers have found that DGLA plays multiple roles against inflammation and proliferation diseases [18], while ARA can enhance the visual and cognitive abilities of infants [19]. EPA and DHA both show beneficial properties against cancer, cardiovascular diseases, inflammation, asthma and allergic diseases, and DHA, especially, is beneficial to neurological development of the fetus and the newborn [5, 20-22]. In addition, VLCPUFAs are found to be capable of preventing other diseases such as hypertension [23], type 2 diabetes [24], diabetic renal diseases [25], rheumatoid arthritis [26], ulcerative colitis [27], Crohn's

disease [28], and chronic obstructive pulmonary disease [29]. However, it has also been reported that the over-consumption of ω-6 VLCPUFAs has led to increases of chronic inflammatory diseases such as nonalcoholic fatty liver disease [30, 31], cardiovascular disease [32], obesity, inflammatory bowel disease [33], rheumatoid arthritis [34], and Alzheimer's disease [35]. It has often been suggested that a deleterious consequence associated with the consumption of ω-6 VLCPUFAs reflects excessive production and activities of ω-6 VLCPUFA-derived eicosanoids, while the beneficial effects associated with the consumption of ω-3 VLCPUFAs reflect the excessive production and activities of ω-3 VLCPUFA-derived docosanoids/eicosanoids. Therefore, the ratio of ω6/ω3 PUFAs has a large influence on their nutritional functions and a low ratio in diet is often recommended for human to stay healthy [32, 36-38].

Humans and animals cannot synthesize VLCPUFAs *de novo* due to the absence of Δ12 desaturase and Δ15 desaturase, which catalyze the formation of linoleic acid (LA, 18:2n-6) and α-linoleic acid (ALA, 18:3n-3), respectively. Therefore, LA and ALA are normally termed essential fatty acids serving as the precursors for biosynthesis of VLCPUFAs in human body. However, the conversion rates of these essential fatty acids to VLCPUFAs like EPA and DHA are very low, varying very much amongst people of different gender and age, and rarely exceeding 4% [39, 40]. Therefore, dietary VLCPUFAs are highly-recommended for human and animals to maintain health [22].

2.2 Biosynthesis of VLCPUFAs

In nature, VLCPUFAs can be synthesized via two distinct pathways. The aerobic pathway involves alternating desaturation and elongation steps of precursor fatty acids such as LA and ALA, whereas the anaerobic pathway utilizes a single multifunctional polyketide-synthase (PKS)-like PUFA synthase to synthesize VLCPUFAs directly from the initial precursor acetic acid.

2.2.1 The aerobic pathway for VLCPUFA biosynthesis

The *de novo* biosynthesis of VLCPUFAs begins with the synthesis of saturated fatty acids catalyzed by fatty acid synthase (FAS). There are two types of FAS in nature. Type I FAS is a single, large and multifunctional polypeptide that mainly occurs in mammals and fungi, while type II FAS in plants and bacteria is a complex process comprising several discrete enzymes [41]. Both types of FAS use acetyl-CoA/ACP and malonyl-CoA/ACP as substrates and catalyze recurring reactions elongating the carbon chain by two carbons at each cycle until palmitic acid (16:0) or stearic acid (18:0) is synthesized (Figure 2-2). Generally, acetyl-CoA and malonyl-CoA are first incoporated

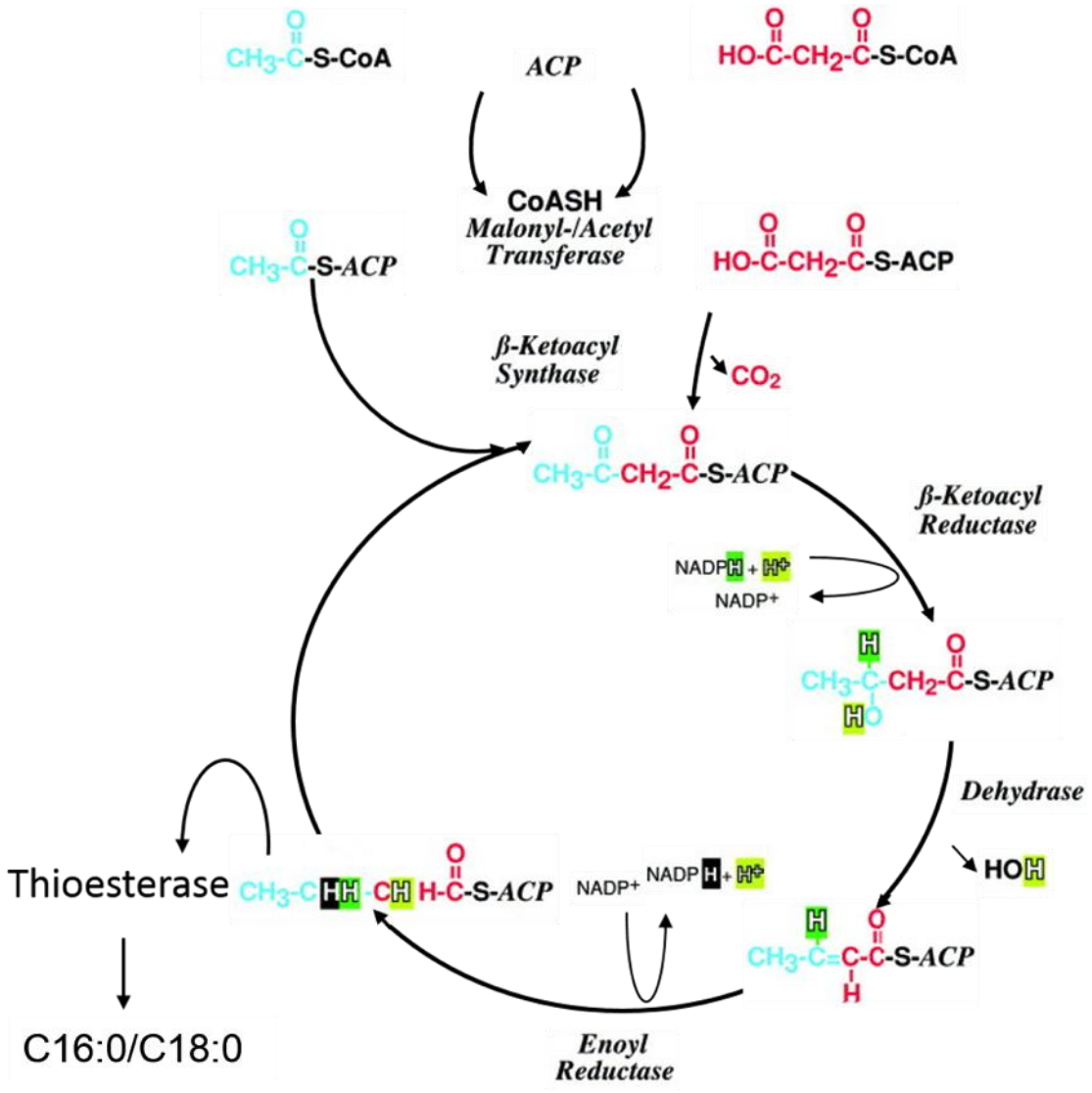

Figure 2-2. Biosynthesis of saturated fatty acids by FAS.

Modified from Smith *et al.* (2003) *Progress in Lipid Research* **42**, 289-317[42].

into acyl carrier protein (ACP), then undergo recurring condensation, reduction, dehyration and reduction reactions catalyzed by β-ketoacyl synthase, β-ketoacyl reductase, dehydrase and enoyl reductase, respectively. Following each cycle of elongation, the acyl chain is elongated by two carbons. After eight or nine elongation cycles, palmitic acid or stearic acid is typically released from ACP by thioesterase. Stearic acid can undergo a number of modifications including desaturation and elongation to form unsaturated fatty acids in the aerobic pathway (Figure 2-3).

The aerobic pathway for VLCPUFA biosynthesis involves alternating elongation and desaturation of precursor fatty acids such as LA and ALA, and mainly occurs in animals, and some eukaryotic microorganisms [43]. Saturated stearic acid (18:0) is sequentially desaturated by Δ9 and Δ12 desaturases giving rise to LA. LA is then converted by Δ15 desaturase to ALA. LA and ALA are the precursors for the biosynthesis of ω-6 and ω-3 VLCPUFAs, respectively. Conventionally, LA and ALA undergo Δ6 desaturation, Δ6 elongation and Δ5 desaturation to form ARA and EPA, respectively. The products of the ω6 pathway can be converted into their ω-3 counterparts by ω3 desaturase. An alternative pathway (Δ8 pathway) exists in some organisms, where LA and ALA are firstly elongated with two carbons and then desaturated by Δ8 desaturase to form DGLA and ETA, respectively. DHA can be synthesized through a sequential Δ5 elongation and Δ4 desaturation of EPA in microorganisms. However, an alternative pathway (Sprecher pathway) involving retro-conversion of a 24-carbon Δ6 fatty acid via β-oxidation is found to be responsible for the production of DHA in mammals [43-46].

2.2.2 The anaerobic pathway for VLCPUFA biosynthesis

In the aerobic pathway for DHA biosynthesis, all involved desaturases consume NADH and require molecular oxygen to introduce double bonds into existing acyl chains. An alternative pathway for DHA biosynthesis exists in some microorganisms that introduces double bonds in the process of acyl elongation [43, 47]. Reactions in this pathway are catalyzed by a polyketide-synthase-like (PKS-like) enzymatic complex called PUFA synthase. The VLCPUFA biosynthesis catalyzed by PUFA synthase starts with the condensing of malonyl-ACP and acetyl-ACP to form a ketoacyl-ACP, followed by reduction of the ketoacyl-ACP to form a hydroxyacyl-ACP, dehydration of the hydroxyacyl-ACP to form an enoyl-ACP, and reduction of the enoyl-ACP to form an acyl-ACP with two additional carbons in the acyl chain. This process is very similar to the elongation of saturated fatty acids by FAS. However, unlike FAS, in some cycles of the reactions by PUFA synthase, the enoyl-ACP does not undergo reduction to form saturated acyl-chain. Instead, it

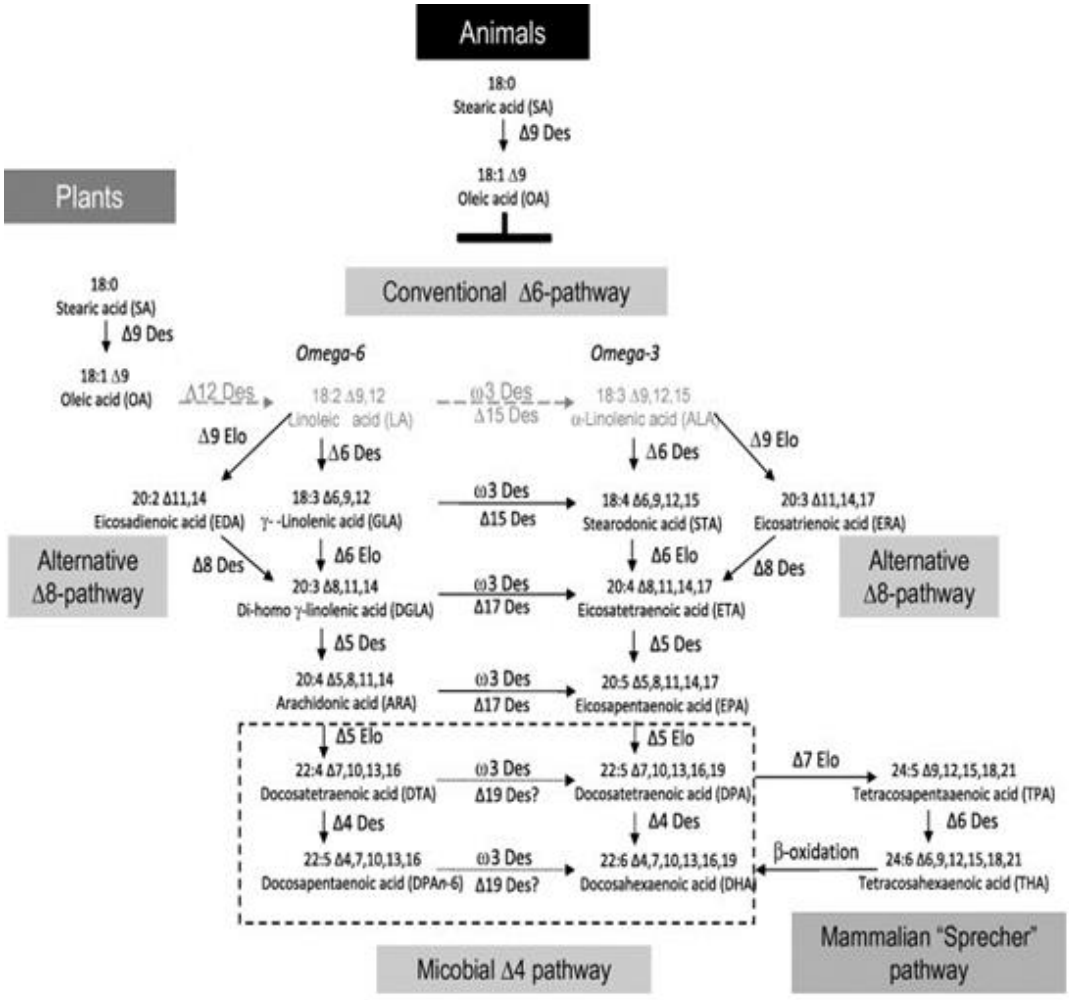

Figure 2-3. The aerobic pathway for VLCPUFA biosynthesis.

Des: desaturase, Elo: elongase. The question marks represent putative enzymes. The light-colored pathway does not exist in animals. Modified from Venegas-Caleron *et al*. (2010) *Progress in Lipid Research* **49** (2): 108-119 [48].

condenses with another malonyl-ACP to form a ketoacyl-ACP with the carbon-carbon double bond retained in the acyl chain. This pathway is independent of oxygen-requiring desaturation to introduce double bonds and is thus called the anaerobic pathway (Figure 2-4).

2.3 Pathways for the assembly of fatty acids in different glycerolipids

The destination of freshly-synthesized fatty acids is either degradation or incorporation into various glycerolipids or sphingolipids. Glycerolipids are lipids with a glycerol backbone linked with fatty acid chains. Based on the absence or presence of a phosphorous headgroup on its *sn*-3 position, glycerolipids can be classified into neutral lipids such as monoacylglycerol (MAG), diacylglycerol (DAG) and triacylglycerol (TAG) and phospholipids such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and their lyso-counterparts. The structures of typical glycerolipids are shown in Figure 2-5. Sphingolipids are lipids with a sphingosine backbone with a fatty acid and a polar head group. As neutral lipid TAG is the major storage lipid in most organisms and phospholipids are the major components of cell membranes, their biosynthesis is very important in the understanding of lipid metabolism. Therefore, the assembly pathways of glycerolipids are emphasized in this study.

2.3.1 Activation of free fatty acid (FFA) to acyl-CoA

After synthesis, free fatty acids (FFA) must be incorporated into different complex lipids such as TAG and phospholipids. To be incorporated, these FFAs much be activated to the form of acyl-CoA by acyl-CoA synthetase (ACS) in an ATP-dependent reaction (Figure 2-6) [49]. SFAs such as palmitic acid or stearic acid are synthesized by either type I or type II FAS in the cytosol of animal and microorganism cells or in the plastid of plant cells. In animals, end products of FAS are palmitoyl-ACP or stearoyl-ACP. The ACP moiety is removed by acyl-ACP thioesterase to produce FFAs which are then activated by ACS. In fungi including yeast, acyltransferase transfers acyl-group from acyl-CoA to the glycerol backbone. However, in plants and most bacteria, acyl-ACP itself can serve as a donor for glycerolipid synthesis [49, 50]. VLCPUFAs synthesized via the aerobic pathway mostly go through a final desaturation step, which also generates fatty acids in an acyl-CoA form. However, VLCPUFAs synthesized by PUFA synthase are released as FFAs, which need an acyl-CoA synthetase to be activated [49, 51]. In addition to glycerolipid assembly, β-oxidation of the fatty acids also happens in the form of acyl-CoA in peroxisomes. However, the

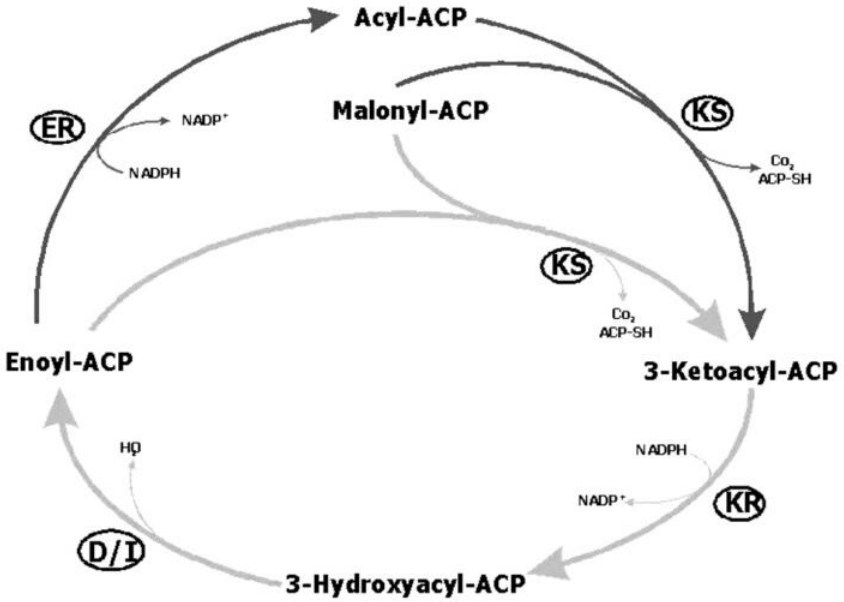

Figure 2-4. Anaerobic pathway for the PUFA synthesis.

KS, 3-ketoacyl synthase; KR, 3-ketoacyl-ACP reductase; D/I, dehydrase/isomerase; ER, enoyl reductase [43].

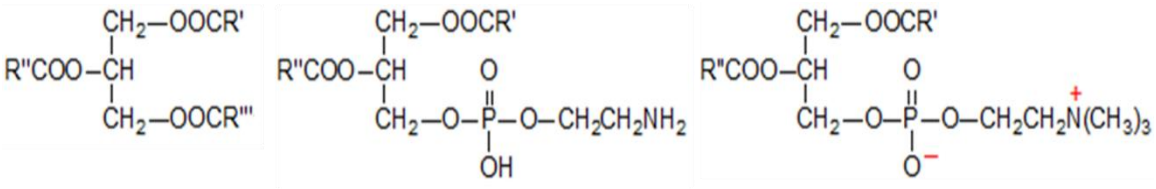

Figure 2-5. Chemical structures of typical glycerolipids.

R represents an alkyl chain.

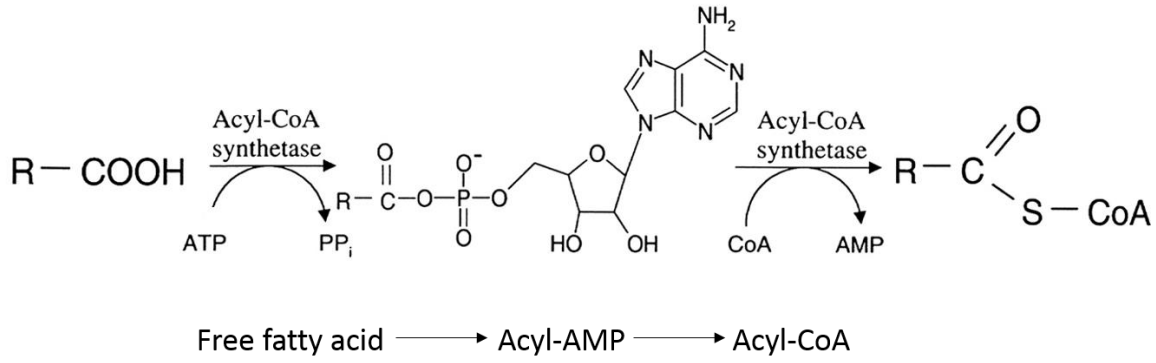

Figure 2-6. Synthesis of acyl-CoA from FFA by ACS.

synthesis of eicosanoids and docosanoids can take place directly in the oil-body using FFAs instead of acyl-CoAs as substrates [52].

2.3.2 Biosynthesis of TAG

TAG is a major storage lipid in nature except in a few living organisms like jojoba plant and small crustacean copepods which synthesize wax esters as major storage lipids [53, 54]. Different pathways for TAG assembly are shown in Figure 2-7. In all pathways, DAG is the direct precursor for the synthesis of TAG. Traditionally, TAG is synthesized in a *de novo* pathway called the Kennedy pathway [55]. In the Kennedy pathway, glycerol-3-phosphate (G3P) is acylated with an acyl group at the *sn*-1 position by G3P acyltransferase (GPAT) to form lysophosphatidic acid (LPA). LPA is then acylated with another acyl group at the *sn*-2 position by LPA acyltransferase (LPAAT) to form phosphatidic acid (PA). The phosphate group of PA is then removed by PA phosphatase (PAP) to form *de novo* DAG, which can be converted into TAG by acylating another acyl group at the *sn*-3 position by DAG acyltransferase (DGAT). A different pathway for TAG synthesis was discovered recently in plants where the precursor DAG is derived from PC [56]. In this pathway, a new DAG molecule is formed by transferring the choline group of PC to the *sn*-3 position of a *de novo* DAG molecule catalyzed by phosphatidylcholine: diacylglycerol phosphocholine transferase (PDCT), resulting in the exchange of fatty acid composition between PC and DAG, but no net production of DAG (DAG1 + PC1→ PC2 + DAG2). The PC-derived DAG is then converted into TAG by DGAT. Another pathway for TAG synthesis is the MAG pathway (MAG→DAG→TAG), which is mainly found in mammalian small intestine [57].

In addition, other pathways exist in TAG biosynthesis but are considered as less important. For example, diacylglycerol transacylase (DGTA) serves an alternative pathway to convert DAG into TAG by transferring one acyl group from one DAG to another, resulting in one molecule of TAG and one molecule of MAG [58]. Enzymes with DGTA activity are found in animals, plants and yeast. Another example is dihydroxyacetone-phosphate (DHAP) pathway to synthesize LPA. DHAP is added with an acyl group from acyl-CoA by DHAP-acyltransferase (DHAP-AT) to form 1-acyl-DHAP, which can be converted into LPA by reduction [59]. It is believed that the DHAP pathway can contribute largely to LPA synthesis when glucose is depleted in a cell [59]. Apart from the acyl-CoA pool, DAG can also utilize PC as an acyl donor, which transfers the *sn*-2 acyl chain to the *sn*-3 position of DAG catalyzed by phospholipid: diacylglycerol acyltransferase (PDAT), forming one molecule of TAG and one molecule of LPC.

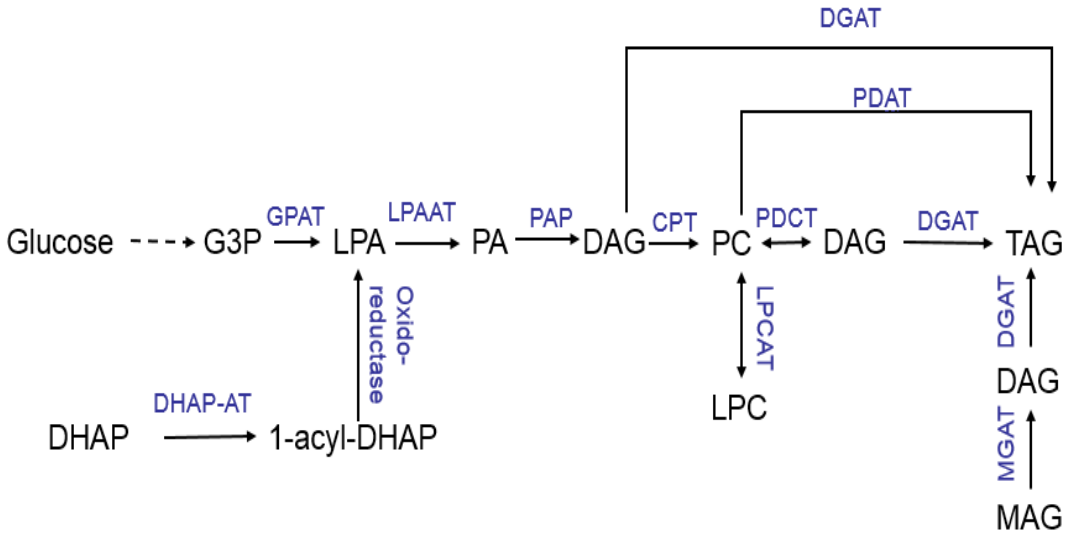

Figure 2-7. TAG synthesis pathways.

GPAT, glycerol-3-phosphate acyl transferase; LPAAT, lyso-phosphatidic acid acyl transferase; PAP, phosphatidic acid phosphatase; CPT, cholinephosphotransferase; DGAT, diacylglycerol acyltransferase; PDAT, phospholipid: diacylglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol cholinephosphate transferase; LPCAT, lysophosphatidylcholine acyltransferase; DHAP-AT, dihydroxyacetone-phosphate acyltransferase; MGAT, monoacylglycerol acyltransferase.

2.3.3 Biosynthesis of phospholipids

PC and PE are the two major phospholipids in eukaryotic cells comprising about 50 and 25% of phospholipid mass, respectively [60]. PC is especially important in lipid anabolism because it is the main recipient for newly-synthesized acyl groups and is the substrate for producing VLCPUFAs in the aerobic pathway. Figure 2-8 shows the various pathways for phospholipid biosynthesis as well as acyl-editing involved in the biosynthesis of PC.

PC can be synthesized by two distinct pathways, namely, CDP-DAG and the Kennedy pathways. A large part of PA is converted into CDP-DAG by CDP-DAG synthase (CDS), which is the precursor for many phospholipids like PI, cardiolipin (CL), PE and PC. In the CDP-DAG pathway, a serine molecule is firstly incorporated into CDP-DAG to form PS, which is decarboxylated into PE. PE can be then converted into PC by three sequential methylation reactions. As a competitive pathway against PS synthesis, PI is synthesized by incorporating one inositol into CDP-DAG. In the Kennedy (*de novo*) PC synthesis pathway, choline is firstly phosphorylated by choline kinase (CK). CDP-choline is then formed with the presence of cytosine triphosphate (CTP). The final step is catalyzed by cholinephosphotransferase (CPT), where PC is synthesized by transferring the choline-P group of CDP-choline to DAG, with the releasing of CMP. PE can also be *do novo* synthesized in a similar way with the presence of ethanolamine [61, 62].

An important flux of fatty acids to PC is for acyl-editing, where the *sn*-2 acyl group on PC is edited via removal and re-incorporation catalyzed by phospholipase A2 and LPCAT [63]. In this pathway, an acyl group is incorporated into PC and goes through modification (desaturation, hydroxylation, etc.) before being released back into the acyl-CoA pool [64]. The desaturation steps in the aerobic pathway of VLCPUFA biosynthesis actually occur on PC during acyl editing. New reactions in PC acyl-editing have been discovered recently in yeast and plants with two newly-identified enzymes: lysophosphatidylcholine transacylase (LPCT) and glycerol-3-phosphocholine acyltransferase (GPCAT) [65-67]. LPCT catalyzes the acyl-transfer between two LPC molecules, resulting in one PC molecule and one glycerol-3-phosphocholine (GPC) molecule. GPCAT catalyzes the re-incorporation of an acyl group into GPC to form LPC. The combined action of LPCT and GPCAT provides a new route of PC re-synthesis after its deacylation. Acyl-editing on PC plays an important role in the aerobic synthesis of PUFAs and can thus affect the fatty acid profile on TAG. It is reasonable to speculate that acyl-editing is essential in PUFA-producing organisms. In non-PUFA-producing organism, however, acyl-editing may not be essential, as found in yeast [68].

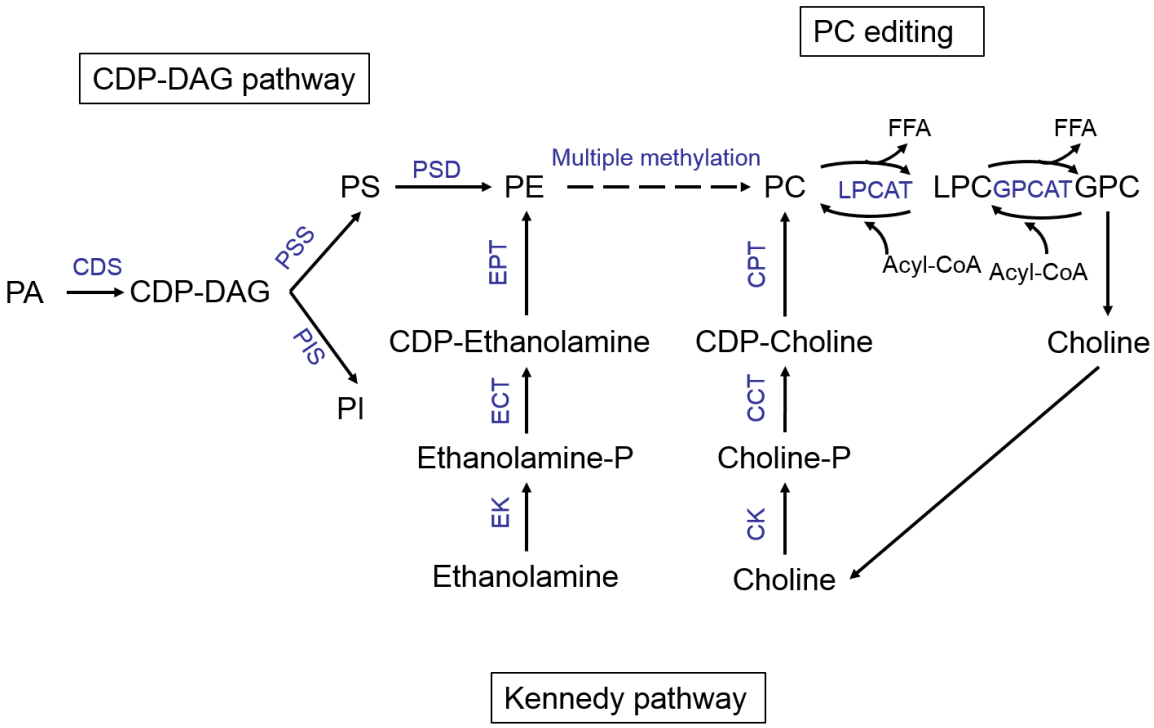

Figure 2-8. Pathways for phospholipid biosynthesis.

CDS, CDP-DAG synthase; PSS, PS synthase; PIS, PI synthase; PSD, PS decarboxylase; EK, ethanolamine kinase; ECT, phosphoethanolamine cytidylyltransferase; EPT, ethanolamine phosphotransferase; CK, choline kinase; CCT, phosphocholine cytidylyltransferase; CPT, choline phosphotransferase; LPCAT, LPC acyltransferase; GPCAT, GPC acyltransferase; only the most important enzymes are listed for reactions catalyzed by multiple enzymes.

In summary, the major pathways for glycerolipid biosynthesis are as follows: i) TAG can be synthesized in two different reactions, one is the *sn*-3 acylation of DAG utilizing an acyl-CoA, catalyzed by DGAT, while the other is the direct transfer a fatty acid from PC to DAG, catalyzed by PDAT; ii) DAG can also be synthesized by two pathways, one is the *de novo* pathway starting from the acylation of G3P, while the other is the PC-derived pathway catalyzed by PDCT; iii) PC can be synthesized by two pathways as well, one is the CDP-DAG pathway ending in multiple methylation of PE and the other is the Kennedy (*de novo*) pathway starting from activation of free choline; and iv) Acyl-editing on PC driven by LPCAT plays an important role in determining the final fatty acid composition of TAG. In addition, there are several enzymes that can influence the incorporation of fatty acids into TAG, such as DGAT, PDAT, PDCT, CPT, and LPCAT. Studying these enzymes can contribute to our understanding of the incorporation of VLCPUFAs in glycerolipids.

2.4 Production of VLCPUFAs in *Thraustochytrium* sp. 26185

As marine fish are under intense pressure from over-exploitation and ocean pollution, it is imperative to explore alternatives to fish oil for VLPUFAs, such as transgenic plants and VLCPUFA-producing microorganisms. Microorganisms that can naturally-accumulate large amounts of VLCPUFAs are ideal sources of these fatty acids through enhanced fermentation and metabolic engineering. In addition, as the acyl flux is important for effective production of a high levels of VLCPUFAs, studying the process in elite VLCPUFA-producing microorganisms would provide a good understanding of the mechanism for the effective VLCPUFA assembly in native species and help formulate strategies for improved production of these fatty acids in transgenic plants.

Thraustochytrids is a family of VLCPUFA producing microorganisms that are usually characterized by their morphological and developmental characteristics, such as sorus development, ectoplasmic net, shape of zoospores and so on. *Schizochytrium* and *Thraustochytrium* are two main VLCPUFA-producing genera in *Thraustochytriidae* family, belonging to the order *Labyrinthulida*, class *Labyrinthulea* and phylum *Labyrinthulomycota* [69]. Many, though not all, *Schizochytrium* and *Thraustochytrium* species have been found to be capable of producing large amounts of VLCPUFAs such as *S. limacinum*, *S. aggregatum*, *T. aureum*, *T.* sp, *T. roseum*, etc. [6]. Notably,

growth conditions are important for these species to produce a substantial amount of EPA and DHA [70-72].

Because of the significant VLCPUFA-producing capacities of *Thraustochytrids*, the study of the fatty acid biosynthesis by *Thraustochytrids* has made great progress. In 2001, a PUFA synthase pathway was firstly-discovered in *Schizochytrium*, revealing for the first time an anaerobic DHA biosynthesis pathway in nature [47]. In the same year, a Δ4 desaturase was discovered in *Thraustochytrium* sp. 26185, validating the existence of an aerobic Δ4 desaturation pathway for DHA synthesis [73]. Since then, various genes and enzymes involved in the biosynthesis of VLCPUFAs were identified in these two genera, such as Δ5 desaturase [74], Δ5 elongase [75], Δ6 elongase, Δ9 elongase [76], and Δ12 desaturase [77]. These findings indicate the possible coexistence of aerobic and anaerobic pathways for VLCPUFA synthesis in *Thraustochytrids*. However, which pathway is more important in *Thraustochytrids* has only been rarely investigated. Interestingly, a series of unusual odd-chain SFAs and PUFAs were also identified in some species of *Thraustochytrids* [78], leading to questions about the biosynthetic mechanism of odd-chain fatty acids. In addition, the assembly process of VLCPUFAs after synthesis is also not well understood. It is known that DHA and EPA are mainly located in phospholipids and TAGs in *Thraustochytrids.* In *Schizochytrium*, it was found that VLCPUFAs synthesized by PUFA synthase are released as FFAs [49] and then sequentially incorporated into glycerolipids, possibly through various pathways. Recently, a lysophospholipid acyltransferase (LPAT) that preferentially incorporates DHA into PC and PE was identified in *Thraustochytrids* [79], indicating the involvement of the acyl editing pathway in phospholipid assembly. Nevertheless, more work is required for a better understanding of VLCPUFA assembly in *Thraustochytrids*.

In recent years, investigations of VLCPUFA production in *Thraustochytrids* has made tremendous progress. For example, higher dissolved oxygen in the medium using air-lift bioreactors instead of stirred tank vessels was found to promote the production of DHA by *Thraustohytrium sp.* BM2 to a DHA level of 27.57 g/L [80]. It was also found that higher total nitrogen concentration in the medium could promote the production of DHA by *Thraustohytrium* sp. 26185 [81]. The cultivation conditions of *Thraustochytrium striatum* have subsequently been optimized, and include; high C/N ratio, 100% seawater, and pH 6-7 for improved fatty acid accumulation [82]. A strain of *Thraustochytrium, kinnei* isolated from the Antarctica peninsula was found to produce the highest amount of DHA at 10°C [83]. Addition of proper amounts of organic salts, such as sodium acetate,

pyruvate, citrate and malate or inorganic phosphates, can dramatically increase lipid and DHA accumulation in a *Thraustohytrium* sp. strain isolated from South India. Fermentation of *Thraustochytrids* using different carbon sources has also been investigated. *Thraustochytrium kinney* VAL-B1 can produce DHA up to 2.53 g/L using residual glycerol of biodiesel production from canola oil as carbon source [84]. Metabolic engineering strategies have also been applied for enhanced production of VLCPUFAs from *Thraustochytrids*. For example, over-expression of the *SOD1* gene, which alleviates the growth stress from reactive oxygen species, was found to increase VLCPUFA production by 1.37-fold in *Thraustohytrium sp*. PKU#Mn4 [85]. In one study, the acyltransferase domain of the PUFA synthase in *Schizochytrium sp.* was replaced by an acyltransferase domain of a PUFA synthase from *Shewanella*, and an increased VLCPUFA content was observed [86]. In another study, an adapted *Schizochytrium sp.* strain increased lipid production by 53.31% to 80.14g/L under high salinity conditions [87]. The lipid extraction method from VLCPUFA-producing microorganisms has also been investigated in recent years. A chloroform-free, ionic, liquid-mediated method for efficient extraction of DHA from *Thraustochytrium* was developed [88]. An ionic liquid consisting of 1-ethyl-3-methylimidazolium ethylsulfate mixed with methanol was shown to efficiently disrupt the cell wall of *Thraustochytrium* thereby facilitating the recovery of DHA. In addition, osmotic shock was found more helpful than liquid nitrogen grinding, bead vortexing and sonication in cell disruption of *Thraustochytrium* and *Schizochytrium* for efficient lipid extraction [89]. Extracting VLCPUFA-containing oil from *Schizochytrium* using supercritical CO_2 was also found to be an efficient green technology [90].

Even though production of VLCPUFAs by *Thraustochytrids* via enhanced fermentation has seen great progress, it is believed that a better understanding of the biosynthetic mechanism of VLCPUFAs and the acyl flux pathways during glycerolipid assembly would greatly boost the development of *Thraustochytrids* lipids as an alternative to fish oil for VLCPUFAs.

2.5 Flux analysis of fatty acid assembly

Over the past decade, the acyl flux during glycerolipid assembly in plants has been intensively investigated in a few studies where radioactive labeled tracers were applied. The results shed light on our understanding of glycerolipid assembly in VLCPUFA-producing microorganisms.

2.5.1 Radioactive tracers: a powerful tool for flux analysis

Fatty acid flux among different phospholipids and neutral lipids determines the fatty acid profile in the final storage lipid TAG. Therefore, it is of vital importance to understand the relative flux efficiency of VLCPUFAs in these phospholipids and neutral lipids. Radioactive tracers are powerful tools for the flux analysis because the kinetics of a labeled substrate can be measured during its metabolism from initial precursors, through intermediates, to final products in metabolic pathways.

There are two ways to perform tracing experiments, namely continuous steady-state labeling and pulse-chase labeling. The former method is done by providing a labeled substrate continuously over a time-course. The relative time for a labeled substrate to accumulate in each metabolite is related to the order of that metabolite in a pathway. Thus, the metabolite with the shortest lag time (the time needed to accumulate detectable amount of radioactivity) is the initial precursor while the metabolite with the longest lag time is the final product in a pathway. The latter approach is performed by providing a labeled substrate for only a short period of time (pulse) and then providing the same unlabeled substrate over a long time-course (chase). Thus, metabolites that lose labeling earlier during the chase period indicate the early precursors, while metabolites that gain labeling later indicate the late products in a pathway [91].

In a steady-state labeling experiment, an appropriate length of a time-course is critical. If intermediate pools of a metabolic pathway reach saturation with labeled metabolites during a long-time course of labeling, the relative amount of labeling between intermediate metabolites reflects the pool size of each metabolite, rather than the production order of labeled metabolites in the pathway. Therefore, in order to analyze the flux pathway, a time-course with time points short enough at the initial stage of labeling is required; in order to determine the pool size of each metabolite, a time-course with time points long enough at later stages of labeling is necessary. In a pulse-chase labeling experiment, the time length of labeling is also critical. The pulse time should be long enough so that enough radioactive tracers can be incorporated for proper analysis, but short enough so that the tracer does not get incorporated into the final product.

2.5.2 Flux analysis of fatty acid assembly using radioactive tracers

Two radioactive tracers are mostly used for flux analysis during glycerolipid assembly: ^{14}C-acetate and ^{14}C-glycerol. The acyl flux can be measured using ^{14}C-acetate as it is mainly utilized for fatty

acid synthesis or fatty acid elongation after being activated into acetyl-CoA. Newly-synthesized fatty acids can go through various pathways such as biosynthesis of *de novo* DAG, phospholipids and TAG. Even though the radiolabeled fatty acids can participate in sphingolipid synthesis and enter into β-oxidation, the majority of radioactivity is often found in phospholipids and TAG in robustly-growing cells. The backbone flux during glycerolipid assembly can be measured using ^{14}C-glycerol as it goes into the G3P acylation pathway after phosphorylation. Glycerol is firstly-utilized to synthesize *de novo* DAG, which is then converted either to TAG or to PC for other DAG synthesis. Therefore, ^{14}C-glycerol is particularly useful for determining the relative flux of TAG synthesis from *de novo* DAG or PC-derived DAG.

The power of radiolabeling in the analysis of acyl flux was firstly-revealed in plants. In 2007, a labeling experiment [56] performed on pea leaves revealed no precursor-product relationship between ^{14}C-acyl-PA and PC at very early time points. Also, radioactive PC molecules at early time points possessed >90% of newly-synthesized ^{14}C-18:1 and ^{14}C-16:0 acyl groups along with unlabeled acyl groups (18:2, 18:3, or 16:0). In the ^{14}C-glycerol labeling experiment, labeled PC molecules were seen to be highly-enriched with 18:2, 18:3, and 16:0 fatty acids, but not 18:1, the major product of plastid fatty acid synthesis. These results indicated that most newly-synthesized acyl groups are not immediately utilized for PA synthesis but incorporated directly into PC through an acyl editing mechanism. Additionally, the acyl groups removed by acyl editing are largely used for the net synthesis of PC through G3P acylation. In 2009, a labeling experiment [92] performed on soybean embryos detected two DAG pools with different fatty acid compositions, *de novo* DAG and PC-derived DAG. It was found that DAG used for TAG synthesis was mostly derived from PC, whereas *de novo* DAG was mostly used for PC synthesis. In 2011, a labeling experiment performed on *Arabidopsis* seeds expressing castor fatty acid hydroxylase [93] found that *de novo* synthesized DAG containing unusual FAs could not be efficiently converted into PC, resulting in low quantities of unusual FAs accumulating in TAG as well as a decreased overall synthesis of oil. In 2014, a similar labeling experiment [64] utilizing ^{14}C-acetate and ^{14}C-malonate as tracers discovered that acetyl-CoA carboxylase activity was largely decreased in transgenic plants, resulting in a decreased amount of newly-synthesized fatty acids. These results indicate that nascent fatty acids go through three pathways to participate in glycerolipid synthesis. The major flux for nascent fatty acids is direct esterification to the *sn*-2 of PC for acyl-editing. In addition, nascent fatty acids along with recycled acyl-CoAs can be acylated with G3P for the *de novo* DAG synthesis.

Finally, nascent fatty acids can be used for direct *sn*-3 acylation of DAG to generate TAG. DAG used for TAG synthesis is mostly derived from PC, whereas *de novo* synthesized DAG is mostly used for PC synthesis. Acyl-groups on PC for editing come from two sources: acyl-CoAs previously acylated by LPCAT from the acyl-CoA pool and acyl-groups of DAG to PC catalyzed by CPT. The findings yielded from radiolabeling experiments in plants have greatly inspired researchers who want to understand the biosynthesis and assembly of VLCPUFAs in *Thraustochytrium* sp. 26185 where little is known about the synthesis and flux of DHA.

This thesis research was conducted in three studies to test the hypothesis and to meet the objectives. Each study is presented independently in the following three chapters.

Study 1 in Chapter 3: Genomic analysis of genes involved in the biosynthesis of very long chain polyunsaturated fatty acids in *Thraustochytrium* sp. 26185.

Study 2 in Chapter 4: Analysis of the biosynthetic process of fatty acids in *Thraustochytrium* sp. 26185.

Study 3 in Chapter 5: Very long chain polyunsaturated fatty acids accumulated in triacylglycerol are channeled from phosphatidylcholine in *Thraustochytrium* sp. 26185.

3. Genomic analysis of genes involved in the biosynthesis of very long chain polyunsaturated fatty acids in *Thraustochytrium sp.* 26185[1]

In order to understand how fatty acids are synthesized and incorporated in the glycerolipids in *Thraustochytrium* sp. 26185, this study started with sequencing the entire genome of *Thraustochytrium* sp. 26185. By homologous comparison of known genes with annotated genes in the genome, various gene-encoding enzymes involved in the biosynthesis and assembly of VLCPUFAs were identified. Findings in this study provided a genomic basis for later studies in this thesis.

3.1 Abstract

Thraustochytrium sp. 26185 is a marine protist that can produce a large amount of docosahexaenoic acid (DHA, 22:6n-3), an ω3 very long chain polyunsaturated fatty acid (VLCPUFA) of nutritional importance. However, the mechanism of how this fatty acid is synthesized and assembled into the storage lipid triacylglycerol is unclear. Here we report sequencing of the whole genome and genomic analysis of genes involved in the biosynthesis and assembly of the fatty acids in this species. Genome sequencing produced a total of 2,418,734,139 bp clean sequences with about 62-fold genome coverage. Annotation of the genome sequence revealed 10,797 coding genes. Among them, 10,216 genes could be assigned into 25 KOG classes, where 451 genes were specifically assigned to the group of lipid transport and metabolism. Detailed analysis of these genes revealed co-existence of both aerobic pathway and anaerobic pathways for the biosynthesis of DHA in this species. However, in the aerobic pathway, a key gene encoding stearoyl Δ9 desaturase, introducing the first double bond to long chain saturated fatty acid 18:0, was missing from the genome. A genomic survey of genes involved in the acyl trafficking among glycerolipids showed that, unlike plants, this protist did not possess phosphatidylcholine: diacylglycerol cholinephosphotransferase, an important enzyme in bridging two types of glycerolipids, diacylglycerols (DAG) and

[1] The work in this chapter was published: Xianming Zhao, Dauenpen Meesapyodsuk, Cunmin Qu and Xiao Qiu (2016) "Genomic analysis of genes involved in the biosynthesis of very long chain polyunsaturated fatty acids in *Thraustochytrium* sp. 26185." Lipids, 51:1065-1075. This paper is presented in the thesis with the permission of all co-authors.

phosphatidylcholines (PC). These results shed new insight on the biosynthesis and assembly of VLCPUFA in the *Thraustochytrium* sp. 26185.

3.2 Introduction

Very long chain polyunsaturated fatty acids (VLCPUFA) such as arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are crucial for human health and wellbeing. Dietary supplementation of these fatty acids has shown that they can improve performance of eyes, brain and immune systems, and provide protection against chronic diseases such as cardiovascular disorders, inflammatory diseases and metabolic syndrome [5]. Many clinical and animal studies demonstrate that VLCPUFA improve neural development, decrease inflammation, promote angiogenesis, and prevent atherothrombosis through the lipid mediators derived from VLCPUFA by a variety of enzymes, such as cyclooxygenases, lipoxygenases and cytochrome P450 enzymes [94-97].

De novo biosynthesis of VLCPUFA is believed to occur only in microorganisms and go through two distinct pathways [43]. The aerobic pathway involves alternating elongation and desaturation steps to introduce double bonds and to extend the acyl chain. Desaturations are catalyzed by a variety of desaturases including front-end and methyl-end desaturases and require molecular oxygen as a cofactor for the oxidation. Elongations are catalyzed by an elongase complex of four catalytic enzymes with substrate specificity primarily defined by the condensing enzyme. For instance, in the aerobic pathway of DHA synthesis, saturated stearic acid (18:0) produced primarily by a fatty acid synthase (FAS) goes through a series of desaturation and elongation steps by various desaturases and elongases such as Δ9 desaturase, Δ12 desaturase, Δ15 desaturase, Δ6 desaturase, Δ6 elongase, Δ5 desaturase, Δ5 elongase and Δ4 desaturase [43]. On the other hand, the anaerobic pathway is catalyzed by a polyketide synthase (PKS)-like mega-enzyme called polyunsaturated fatty acid (PUFA) synthase, using acetate as the precursor to synthesize VLCPUFA directly. The double bonds are introduced during the elongation process of acyl chains without molecular oxygen involvement [43].

Thraustochytrium sp. 26185 is a marine protist that can produce a high level of VLCPUFA such as DHA and docosapentaenoic acid (DPA, 22:5n-6) in storage lipid triacylglycerols (TAG) [6, 72]. This source of VLCPUFA has been the subject of trials for use in functional food and animal feeds. However, the biosynthesis and assembly of VLCPUFA in glycerolipids is not well understood. In

2001, the first Δ4 desaturase involved in the biosynthesis of DHA was identified and characterized from this species, providing unambiguous evidence that DHA could be synthesized through the final Δ4 desaturation step in the aerobic pathway [73]. Almost at the same time, a PKS-like PUFA synthase was found for the biosynthesis of DHA in *Schizochytrium sp.* 20888, a closely related species to *Thraustochytrium* sp. 26185 [47, 49, 98]. This result has raised questions about whether an anaerobic pathway also exists for VLCPUFA synthesis in *Thraustochytrium* sp. 26185, and if it does, which pathway is more important for the DHA biosynthesis. To answer these questions, we attempted to sequence the entire genome of *Thraustochytrium sp.* 26185. The genome sequences *de novo* assembly and annotation revealed the genome size of *Thraustochytrium* sp. 26185 at about 38.6 Mb with a GC content of 63%, and 10,797 coding genes. Genomic analysis of these genes showed that both aerobic and anaerobic pathways for the biosynthesis of VLCPUFA indeed co-exist in this species. However, in the aerobic pathway, a gene encoding stearoyl Δ9 desaturase for introducing the first double bond to long chain fatty acid 18:0 synthesized by Type I fatty acid synthase, was missing from the genome, implying the aerobic pathway might be incomplete and the alternative anaerobic pathway might be mainly responsible for the biosynthesis of VLCPUFA. Additionally, genome sequence analysis of genes involved in acyl trafficking among glycerolipids showed that the fatty acids could be acylated into the glycerol backbone to synthesize both neutral lipids and polar lipids. However, a gene encoding phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), a critical enzyme involved in the acyl trafficking between phosphatidylcholine (PC) and diacylglycerol (DAG), was missing from the genome, implying that DAG, an immediate precursor for the biosynthesis of TAG might not derive from the catalytic process, as seen in plants. These results shed new insights into the biosynthesis and assembly of VLCPUFA in the *Thraustochytrium* sp. 26185, which is instrumental for not only genetic improvement of the production of VLCPUFA in the species, but also for the transgenic production of these fatty acids in heterologous systems, particularly oil seed crops.

3.3 Materials and methods

3.3.1 *Thraustochytrium* cultivation

The strain, T*hraustochytrium* sp. 26185, was purchased from the American Type Culture Collection (ATCC 26185). It was cultured at room temperature in a BY^+ medium containing 0.1% (w/v) yeast extract, 0.1% (w/v) peptone, and 0.5% (w/v) D-glucose in seawater. Seawater was

prepared by dissolving 40 g of sea salts (Sigma-Aldrich, St. Louis, MO, USA) in 1 L of distilled water. The cells growing in mid-log phase were harvested by centrifugation at 2,500×g for 20 min, washed twice with 50 mL of seawater, and used for genomic DNA extraction.

3.3.2 DNA preparation and library construction

Genomic DNA was extracted from *Thraustochytrium* sp. 26185 using E.Z.N.A HP Fungal DNA kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. Sequencing libraries were prepared according to the Illumina Hiseq protocol (Illumina, San Diego, CA, USA). A total of 5 μg of genomic DNA was fragmented using a Branson sonicator and the ends of DNA fragments were repaired according to previously published method [99]. After being end-blunted, ligated with Hiseq adaptors and amplified by polymerase chain reaction (PCR), the products were purified using agarose gel electrophoresis and cloned into a TOPO plasmid to construct DNA libraries for sequencing. The 300 bp library was used for Illumina Hiseq paired-end sequencing, and the 3 kb library was used for Illumina Hiseq mate-pair sequencing. Sequencing of both libraries was performed on a Hiseq 2000 sequencer.

3.3.3 *De novo* assembly

Processing and assembly of the sequenced data was carried out using a *de novo* method as described previously [100-103]. Raw data were filtered out of adaptors, low quality and duplicated reads via FastQC. Reads with quality scores <20 (one error in 100) and length <25bp were all considered as low-quality reads. The *de novo* sequence assembly was performed by SOAPdenovo assembler (http://soap.genomics.org.cn/soapdenovo.html) [104-106]. Contigs were generated by assembling clean paired-end data and scaffolds were then generated after adding clean mate-pair data.

3.3.4 Gene annotation and analysis

The draft genome sequence was annotated according to the standard method of Integrated Microbial Genomes EXPERT Review (IMG-ER) platform [101, 107]. The sequences of coding region were predicted by the Augustus program (http://bioinf.uni-greifswald.de/augustus/) [108] and were confirmed by BLASTP searches with an E-value threshold of 10^{-5} against the gene bank non-redundant protein database [109]. Gene ontology (GO) annotation of each predicted protein sequence was performed using the Blast2GO program (v.2.4.2) with default parameters [110, 111]. The KOG and KEGG pathway annotation were also performed using BLAST searches against the

Eukaryotic Cluster of Orthologous Groups database (http://www.ncbi.nlm.nih.gov/COG/) and Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/), respectively.

3.4 Results

3.4.1 Genome sequencing and *de novo* assembly

To obtain a genome-wide survey of genes associated with the biosynthesis and assembly of VLCPUFA in *Thraustochytrium* sp. 26185, the whole genome was sequenced using Illumina sequencing platform. After cleaning and quality checks, 2×16,399,848 and 2×9,797,372 raw paired-end and mate-pair reads with the length of 101 bp were generated. The Poisson distributions of GC content and sequencing depth, as well as centralized plots of the distributions of sequencing reads with their GC content and depth (Figure 3-1), indicated that no external DNA contamination or sequencing bias was observed, and the libraries generated for sequencing were of high quality. After filtering out low quality data, a total of 2,418,734,139 bp clean sequences were obtained and assembled into 8,130 contigs with 5,449 large contigs (>1000 bp) and the largest contig reached 88,386 bp in length. These contigs were then assembled into 2,250 scaffolds with the largest scaffold being 819,459 bp in length. The calculated genome size of *Thraustochytrium* sp. 26185 was about 38.6 Mb with a GC content of 62.9% and an N rate (sequencing gaps) of 3.3% (Table 3-1).

3.4.2 Genome sequence annotation

To annotate the genome sequences, the assembled contigs and scaffolds were analyzed by the Augustus program with default parameters. In total, 10,797 predicted coding genes were detected, of which 8,360 had homologous hits by BLAST searches. From Gene Ontology (GO) annotation, 3,178 genes were assigned into 46 groups in three main GO categories: cellular component, molecular function and biological process (Figure 3-2). The majority of these genes were assigned to the biological process category, followed by cellular component and molecular function. To better describe the genome composition, the annotated sequences were BLAST searched against the Eukaryotic Clusters of Orthologous Groups (KOG) database. In total, 10,216 genes were assigned to 25 KOG classifications (Figure 3-3). Among the KOG groups, the cluster of signal transduction mechanisms (13.4%) represented the largest group, followed by the groups of general function prediction (12.9%), post-translational modification, protein turnover,

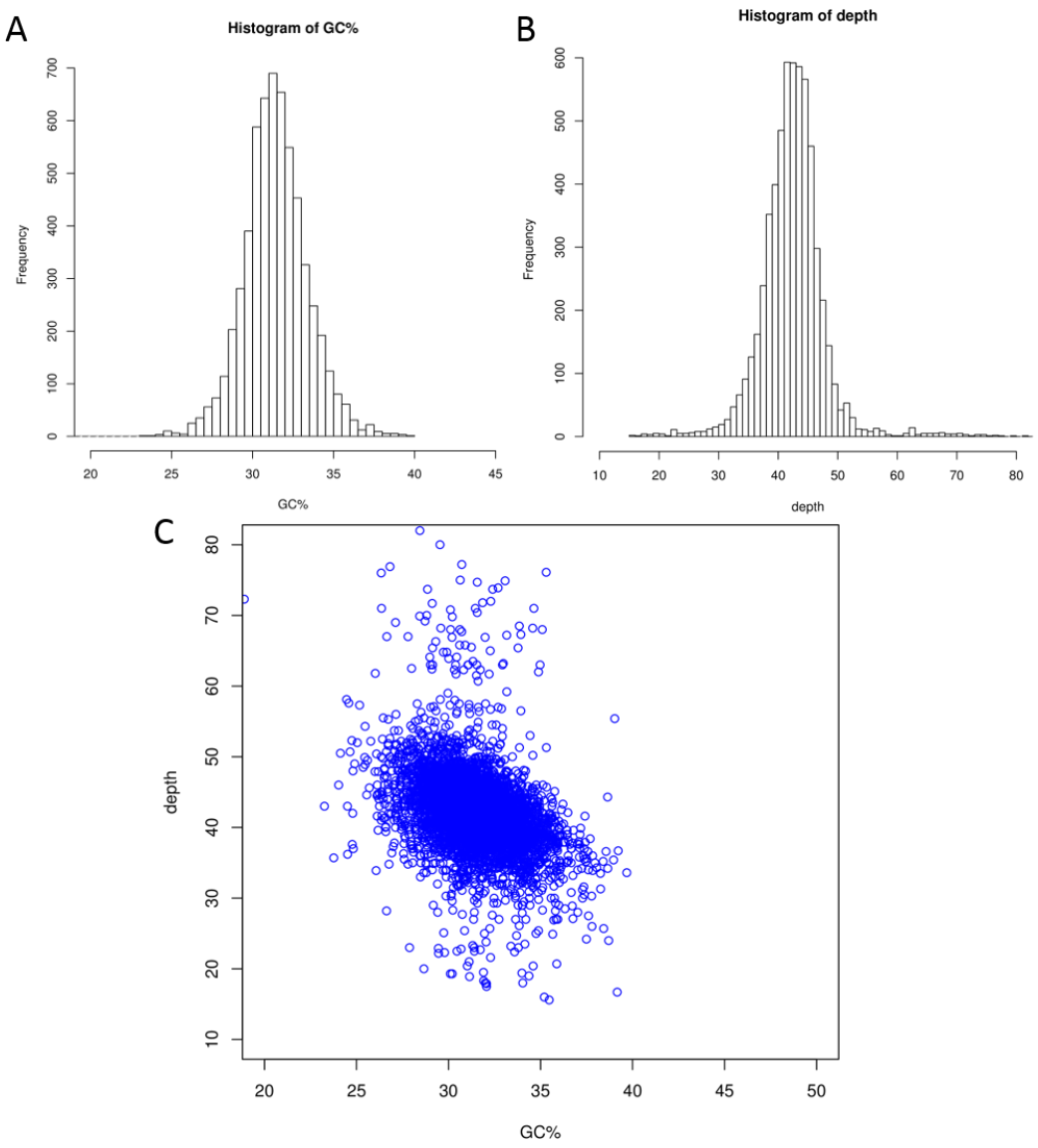

Figure 3-1. GC contents and sequencing depth of the *Thraustochytrium* sp. 26185 genome.

A, GC content distribution of PE sequencing reads; B, sequencing depth of PE sequencing reads; C, GC contents plotted with sequencing depth.

Table 3-1. Statistics of sequencing results.

No. of Scaffolds	2250
Bases in all scaffolds	38,593,592 bp
No. of large scaffolds (> 1000 bp)	421
Bases in large scaffolds	38,192,523 bp
Longest length	819,459 bp
Scaffold N50	238,953 bp
N50 scaffolds	54
Scaffold N90	71,175 bp
N90 scaffolds	160
G + C content	62.952 %
N rate	3.285 %

N50 scaffolds: the number of scaffolds required to cover ≥50% of the genome size when counting down from the largest scaffold;
Scaffold N50: the length of the smallest N50 scaffold;
N90 Scaffolds: the number of scaffolds required to cover ≥90% of the genome size when counting down from the largest scaffold;
Scaffold N50: the length of the smallest N90 scaffold;
N rate: percentage of unidentified nucleotides in the genome.

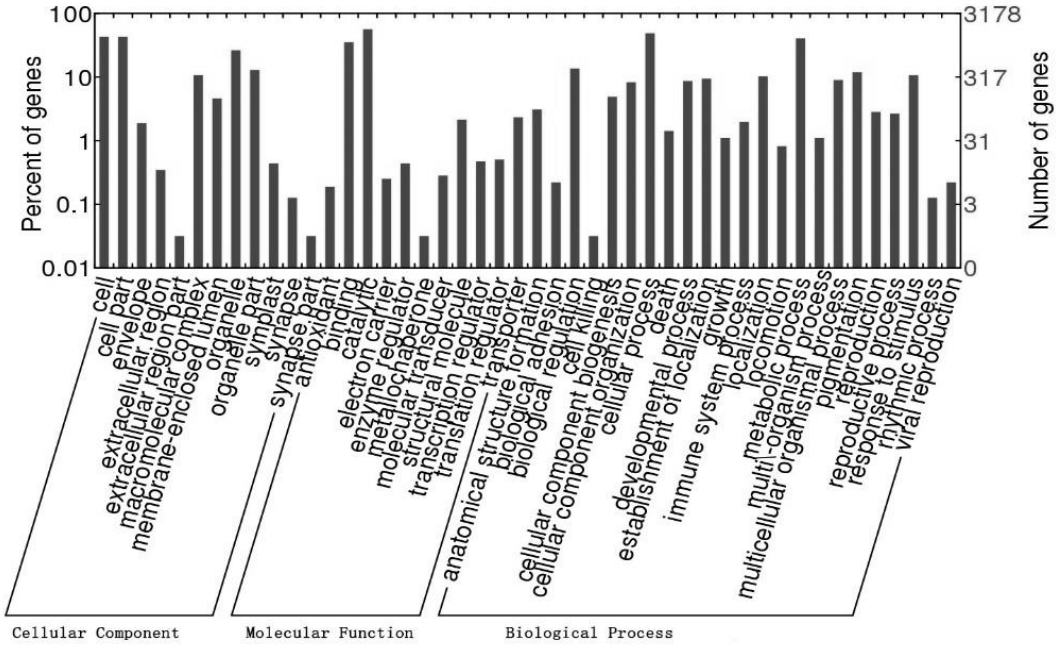

Figure 3-2. Classification of *Thraustochytrium* sp. 26185 genes by GO analysis.

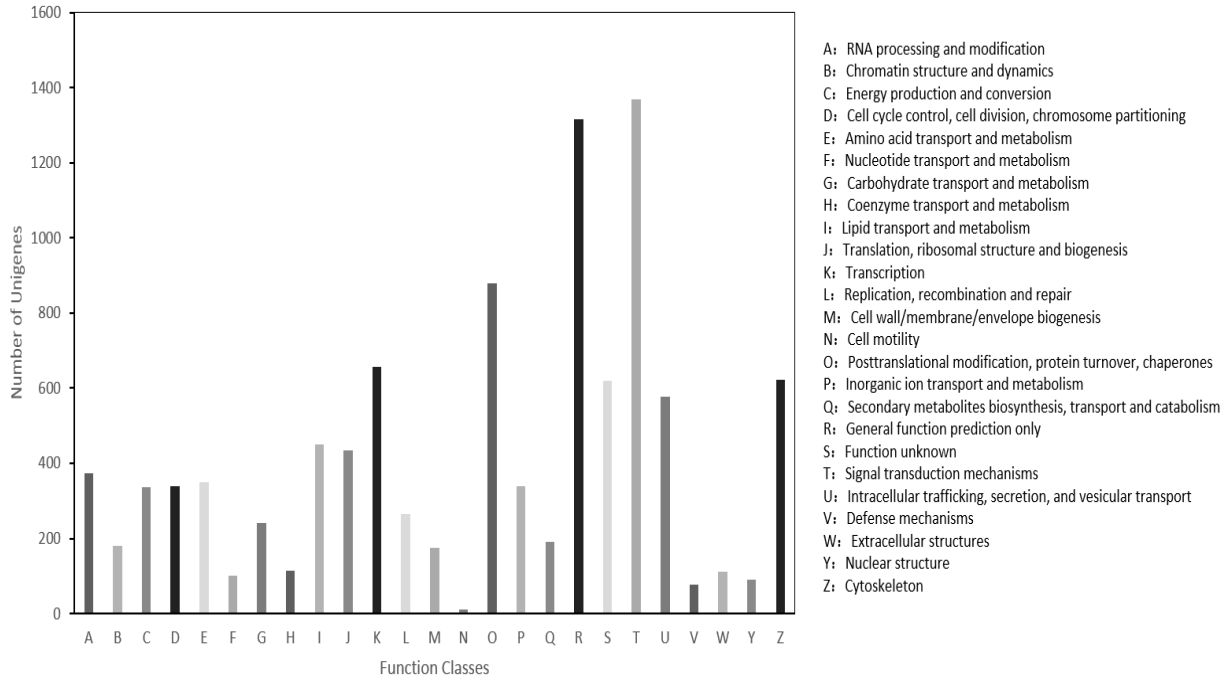

Figure 3-3. Classification of *Thraustochytrium* sp. 26185 genes by KOG analysis.

chaperones (8.6%), and transcription (6.4%). Specifically of interest to this study, there were 451 genes assigned to the group of lipid transport and metabolism. In order to better classify the annotated genes, we BLAST-searched the genes against KEGG pathway database. In total, 2,634 genes were mapped to different pathways, with 135 linked with lipid metabolism (Table 3-2).

3.4.3 Genomic analysis of genes involved in VLCPUFA biosynthesis

In *Thraustochytrium* sp. 26185, like other fungi, the primary fatty acid biosynthesis from acetic acid to long chain saturated fatty acids (16:0 and 18:0) is catalyzed by Type I fatty acid synthase (FAS), a large multifunctional polypeptide with 4,053 amino acids containing four catalytic domains for biochemical reactions: condensation, ketoacyl reduction, hydroxyacyl dehydration and enoyl reduction. In addition, it also possesses an acyl carrier protein (ACP) domain for binding phosphopantetheine prosthetic group to carry an acyl chain. Moreover, a malonyl-CoA ACP transacylase (MAT) domain for catalyzing the conversion of malonyl-CoA to malonyl-ACP was also found in the enzyme. The FAS was highly similar to FAS from *Schizochytrium sp.* with about 90% identity at the amino acid level. Furthermore, a coding region for acetyl-CoA carboxylase (ACC) of 2326 amino acids with biotin carboxylase and biotin-carboxyl-transferase domains was also identified in this species. This enzyme converted acetyl-CoA into malonyl-CoA, which was the rate limiting step of fatty acid synthesis. Additionally, two separate genes encoding MAT were also detected in the genome (Table 3-3).

The biosynthesis of VLCPUFA in the aerobic pathway involves a series of desaturation and elongation reactions (Table 3-3). In *Thraustochytrium* sp. 26185 genome sequence, four putative methyl-end desaturase genes were detected. The functions of two of them were confirmed by heterologous expression in yeast [73]. One encoded a Δ12 desaturase converting oleic acid (18:1-9) to linoleic acid (18:2-9, 12) while the other coded for an ω3 desaturase for converting very long chain ω6 fatty acids to their corresponding ω3 counterparts. In the category of front-end desaturases, the genome possessed three putative Δ6 desaturases, one Δ5 desaturase and one Δ4 desaturase to sequentially introduce three double bonds towards the carboxyl end. Among them, the gene encoding Δ5 desaturase and Δ4 desaturase were previously characterized [73]. All these front-end desaturases had a cytochrome b5-like domain at their N-termini. However, no gene encoding any Δ9 desaturase to convert stearic acid (18:0) to oleic acid (18:1-9) was identified in the genome.

In addition, three condensing enzymes (ELO type) were found to be possibly responsible for elongating polyunsaturated fatty acids such as Δ9 desaturated fatty acids linoleic acid (LA, 18:2-

Table 3-2. Genes assigned to lipid metabolism by KEGG analysis.

Lipid Metabolism	135
alpha-Linolenic acid metabolism	4
Arachidonic acid metabolism	3
Biosynthesis of unsaturated fatty acids	8
Steroid hormone metabolism	4
Ether lipid metabolism	6
Fatty acid biosynthesis	10
Fatty acid elongation	4
Fatty acid degradation	20
Glycerolipid metabolism	17
Glycerophospholipid metabolism	28
Linoleic acid metabolism	2
Primary bile acid biosynthesis	2
Secondary bile acid biosynthesis	1
Sphingolipid metabolism	11
Steroid biosynthesis	11
Synthesis and degradation of ketone bodies	4

Table 3-3. Enzymes involved in the VLCPUFA biosynthesis in *Thraustochytrium* sp. 26185.

Category	Name	Protein Length	Accession number of the top hit	Organism	Amino acid identity (%)
FAS	TsFas	4053	ABJ98780	*Schizochytrium sp.*	90
	TsAcc	2326	KUF86608.1	*Phytophthora nicotianae*	44
	TsMt-1	376	AGG56551.1	*Schizochytrium sp.*	76
	TsMt-2	406	XP_003054808.1	*Micromonas pusilla*	46
Desaturase	TsDes12	417	XP_002186139	*Phaeodactylum tricornutum*	440
	TsDesω3	421	XP_002184864.1	*Phaeodactylum tricornutum*	49
	TsDes6-1	542	AEV77089	*Isochrysis galbana*	48
	TsDes6-2	454	XP_003056992	*Micromonas pusilla*	52
	TsDes6-3	510	XP_002783168.1	*Perkinsus marinus 50983*	31
	TsDes5	439	AAM09687	*Thraustochytrium sp.*	100
	TsDes4	519	AAM09688	*Thraustochytrium sp.*	100
Elongase	TsElo6	271	BAM66615	*Thraustochytrium sp.*	100
	TsElo5	276	BAM66614	*Thraustochytrium sp.*	100
	TsElo9	259	XP_005759783	*Emiliania huxleyi*	39
PUFA synthase	TsPfs-A	2813	AAK72879.2	*Schizochytrium sp.*	87

	TsPfs-B	2049	AAK72880	*Schizochytrium sp.*	85
	TsPfs-C	1497	AAK72881	*Schizochytrium sp.*	89
	TsPpt	206	XP_003385372.1	*Amphimedon queenslandica*	42

Note: All genes are defined as top BLAST hit unless otherwise stated.

9,12) and α-linolenic acid (ALA, 18:3-9,12,15), or Δ6 desaturated fatty acids γ-linoleic acid (GLA, 18:3-6,9,12) and stearidonic acid (SDA, 18:4-6,9,12,15) or Δ5 desaturated fatty acids arachidonic acid (ARA, 20:4-5,8,11,14) and EPA (20:5-5,8,11,14,17) (Table 3-3) [112]. One of these elongases was previously characterized in both yeast and plants [113]. The elongation of fatty acids involves four biochemical reactions for ketoacyl-CoA condensation, ketoacyl-CoA reduction, hydroxyacyl-CoA dehydration and enoyl-CoA reduction. Accordingly, one gene was found to encode a ketoacyl-CoA reductase, three genes were identified to encode hydroxyacyl-CoA dehydratase and one gene was detected to encode an enoyl-CoA reductase in the genome.

Taken together, these results indicate that an aerobic pathway exists for the biosynthesis of VLCPUFA in *Thraustochytrium* sp. 26185. However, the pathway might not be complete since a Δ9 desaturase for the introduction of the first double bond into stearic acid was not present in the genome. The Δ9 monounsaturated acid, oleic acid (18:1-9), is the key precursor for the VLCPUFA synthesis in the aerobic pathway.

In an anaerobic pathway for the biosynthesis of VLCPUFA, three large genes without introns encoding three subunits (A, B and C) of a PUFA synthase were found in *Thraustochytrium* sp. 26185 (Table 3-3). Subunit A (TsPfs-A) was 2,813 amino acid long composed of one 3-ketoacyl-ACP synthase domain (KS1) for condensing malonyl-ACP with the existing acyl chain to form 3-ketoacyl-ACP, one malonyl-CoA:ACP acyltransferase domain (MAT) for transferring malonyl-CoA to ACP domains, 8 acyl carrier protein (ACP) domains, one ketoacyl-ACP reductase domain (KR) for reducing keto group of acyl chains and one hydroxyacyl-ACP dehydrogenase (DH1) domain for removing a water molecule and generating a double bond. Subunit B was 2,049 amino acids in length comprising two KS domains (KS2 and KS3), one acyl transferase domain (AT), and one enoyl-ACP reductase domain (ER1) for reducing double bonds. Two KS domains (KS2 and KS3) in Subunit B were located side by side. KS2 had sequence similarity to *E. coli* FabB while KS3 had sequence similarity to PKS chain length factors. This arrangement might be vital for retaining double bonds during the VLCPUFA synthesis. Subunit C was 1,497 amino acids in length consisting of two dehydratase domains (DH2 and DH3) for introducing double bonds, and one ER domain (ER2). Two DH domains (DH2 and DH3) in Subunit C resided also side by side and both shared sequence similarity to *E. coli* FabA. Overall, the PUFA synthase in *Thraustochytrium* sp. 26185 was highly similar to those from other VLCPUFA-producing microbes. In addition, a discrete phosphopantetheinyl transferase (TsPPTase) with 206 amino acids

required for attaching phosphopantetheine prosthetic group to the ACP domains was also identified in the genome.

3.4.4 Genomic analysis of genes involved in the assembly of VLCPUFA

Acyl-CoA synthetase (ACS) is a member of the ligase family that activates free fatty acids to their acyl-CoA forms, playing an important role in enabling free fatty acids to be incorporated into various glycerolipids. In the *Thraustochytrium* sp. 26185, five genes were identified encoding long chain acyl-CoA synthetase (Table 3-4). They all shared similar structure such as an AMP-binding domain "INYTSGTTGAPK" which is commonly found in members of the AMP-binding proteins such as acyl-CoA synthetase [114].

Once being activated into the acyl-CoA form, newly-synthesized fatty acids could be assembled into neutral lipids such as TAG, wax esters and sterol esters or polar phospholipids such as phosphatidylethanolamine (PE) and PC using various acyltransferases. For instance, in the neutral glycerolipid biosynthesis pathway, a fatty acid is first acylated onto *sn*-1 position of glycerol-3-phosphate (G3P) by G3P acyltransferase (GPAT) to form lysophosphatidic acid (LPA), which is further acylated by LPA-acyltransferase (LPAT) to form phosphatidic acid (PA). DAG is then formed via the dephosphorylation of PA by phosphatidic acid phosphatase (PAP). Finally, TAG is synthesized by incorporation of an acyl chain to DAG catalyzed by DAG acyltransferase (DGAT) [59]. Structurally, these acyl transferases belonged to a membrane-bound O-acyltransferase (MBOAT) superfamily with the conserved motifs catalyzing acylation of an acyl-CoA into various acceptors such as G3P, LPA, LPC or alcohol [115]. In *Thraustochytrium* sp. 26185, five genes encoded GPAT and four genes encoded LPAT in the superfamily. In addition, six genes were identified as DGAT or possibly wax synthase (WS) from the superfamily. There was also one gene found to encode PAP (Table 3-5).

In *Thraustochytrium* sp. 26185, two main phospholipids of biological membranes, PE and PC, could be synthesized by two pathways, the backbone-activating pathway (CDP-DAG) and the head-activating (*de novo*) pathway. In the backbone-activating pathway, CDP-DAG is first synthesized from PA and CTP by CDP-DAG synthase (CDS). Two genes from this species were assumed to encode CDS as they both were identified as a member of CTP transferase family and shared a 42% and 45% similarity with CDS from *Zea mays* and *Budvicia aquatic*, respectively. Phosphatidylserine (PS) could be synthesized from CDP-DAG by phosphatidylserine synthase (PSS). One gene from this species was identified to encode PSS, with 534 amino acids long sharing

Table 3-4. Acyl-CoA synthetases in *Thraustochytrium* sp. 26185.

Name	Protein length	Access number of the top hit	Organism	Amino acid identity (%)
TsAcs1	645	WP_045446568	*Tepidicaulis marinus MA2*	43
TsAcs2	642	WP_043949941	*Candidatus Phaeomarinobacter ectocarpi*	44
TsAcs3	642	WP_045446568	*Tepidicaulis marinus MA2*	45
TsAcs4	611	WP_034743799	Hyphomonas chukchiensis	42
TsAcs5	630	WP_045446568	Tepidicaulis marinus MA2	45

Note: All genes are defined as top BLAST hit unless otherwise stated.

Table 3-5. Enzymes involved in VLCPUFA assembly in *Thraustochytrium* sp. 26185.

Category	Name	Length	Accession number of the top hit	Organism	Amino acid identity (%)
Kennedy Pathway for TAG synthesis					
GPAT	TsGpat1	658	XP_001022288	*Tetrahymena thermophila*	32
	TsGpat2	625	EJY87883	*Oxytricha trifallax*	34
	TsGpat3	536	CCA72258.1	*Piriformospora indica*	52
	TsGpat4	717	XP_006657790.1	*Oryza brachyantha*	52
	TsGpat5	855	XP_002900356.1	*Phytophthora infestans*	41
LPAT	TsLpat1	413	CCA22190	*Albugo laibachii*	33
	TsLpat2	381	XP_002139876	*Cryptosporidium muris*	34
	TsLpat3	331	EPY40720	*Angomonas deanei*	38
	TsLpat4	320	XP_002139876	*Cryptosporidium muris*	38
PAP	TsPap	492	XP_010516498	*Camelina sativa*	7
DGAT	TsDgat1	945	XP_002906875.1	*Phytophthora infestans*	36
	TsDgat2	885	XP_002906875	*Phytophthora infestans*	29
	TsDgat3	551	AGF92151	*Thraustochytrium aureum*	62
	TsDgat4	374	KOO21647	*Chrysochromulina sp.*	48
	TsDgat/Ws1	556	AHG91834.1	*Gemmatirosa kalamazoonesis*	29
	TsDgat/Ws2	523	KPA13432	*Candidatus Magnetomorum*	26
CDP-DAG pathway (backbone activating) for phospholipid synthesis					
CDS	TsCds1	142	WP_017038728	*Vibrio genomosp.*	35
	TsCds2	449	XP_008654451.1	*Zea mays*	42
PSS	TsPss	534	XP_001134578	*Dictyostelium discoideum*	37
PSD	TsPsd1	421	ETM50170	*Phytophthora parasitica*	44

PEMT	TsPsd2	318	ETM50170	*Phytophthora parasitica*	43
	TsPemt	864	XP_005535754	*Cyanidioschyzon merolae*	31
De novo pathway (head activating) for phospholipid synthesis					
EK	TsEk	409	XP_002903667	*Phytophthora infestans*	39
CK	TsCk	401	XP_013758326	*Thecamonas trahens*	34
PECT	TsPect	525	CBJ49051	*Ectocarpus siliculosus*	56
PCCT	TsPcct	425	XP_004338083	*Acanthamoeba castellanii*	41
EPT/CPT	TsE/Cpt1	385	XP_001471498	*Tetrahymena thermophila*	32
	TsE/Cpt2	423	KOO33192	*Chrysochromulina sp.*	32

Note: All genes are defined as top BLAST hit unless otherwise stated.

37% sequence identity with the PSS from *Dictyostelium discoideum*. PS could be decarboxylated by phosphatidylserine decarboxylase (PSD) to produce PE. Two genes were identified to encode PSD with 318 and 421 amino acids, respectively. On the other hand, PE could go through three sequential methylation reactions catalyzed by PE methyltransferase (PEMT) to produce PC. One gene in this species was identified to encode PEMT with 864 amino acids. The protein had two PEMT domains, a common feature in PEMT (Table 3-5).

In the head-activating pathway for PC and PE biosynthesis, ethanolamine and choline are firstly activated by their kinases, ethanolamine kinase (EK) and choline kinase (CK), to form phosphoethanolamine and phosphocholine, respectively. Afterwards, they are incorporated with a CMP group by cytidylyltransferases (PECT and PCCT) to form CDP-ethanolamine and CDP-choline, respectively. The final step is catalyzed by ethanolamine phosphotransferase (EPT) or choline phosphotransferase (CPT) to convert CDP-ethanolamine and CDP-choline into PE and PC, respectively. In *Thraustochytrium* sp. 26185, one gene was identified each encoding EK, CK, PECT and PCCT, respectively. Both ATP binding site and active site were found in EK and CK sequences [116], while "HXGH" and "KMSKS" motifs were found in PECT and PCCT sequences, which were highly conserved among the cytidylyltransferase family [117, 118]. There were two genes identified as either EPT or CPT in the genome with 385 and 423 amino acids long, respectively, and both shared 32% sequence identity with those from *Tetrahymena thermophile* and *Chrysochromulina sp.*, respectively (Table 3-4).

Lands cycle [63] plays an important role in the biosynthesis of phosphoglycerolipids in plants by using a lyso-phosphatidylcholine (LPC) acyltransferase (LPCAT) for incorporation of freshly-synthesized fatty acids to PC [119]. In *Thraustochytrium* sp. 26185, there were two LPCAT of 556 and 612 amino acids for acylation of the *sn*-2 position of LPC to form PC. Both shared high amino acid identity (70% and 42%) with LPCAT from *Aurantiochytrium limacinum*, with a conserved acyl-acceptor pocket and a calcium binding site.

In plants, acyl switching between PC and DAG could be catalyzed by phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), whereby DAG with acyl chains modified on PC could be assembled into TAG by DGAT[120]. Surprisingly, BLAST search did not identify any PDCT homologies corresponding to E value of $< 10^{-5}$ in the genome of *Thraustochytrium* sp. 26185.

3.5 Discussion

Thraustochytrium sp. 26185 has long been known to accumulate a large amount of VLCPUFA in its storage lipids. The goal of this research was to survey metabolic pathways for the biosynthesis and assembly of VLCPUFA in this species through genome sequencing and genomic analysis of genes involved in the processes. Genome sequencing produced a total of 2,418,734,139 bp clean sequences with more than 62-fold genome coverage. Annotation of the genome sequence revealed 10,797 coding genes. Among them, 10,216 genes could be assigned into 25 KOG classes where 451 genes were specifically assigned to the group of lipid transport and metabolism.

Analysis of these genes revealed that two pathways for the biosynthesis of VLCPUFA co-exist in this species. The aerobic pathway is more complex involving many different kinds of enzymes, such as fatty acid synthase for the synthesis of long chain saturated fatty acids (16:0 and 18:0), desaturases and elongases for introducing various double bonds towards both front and methyl ends and for elongating fatty acids with various chain lengths (Figure 3-4). On the other hand, the anaerobic pathway is a *de novo* system with fewer chemical reactions using a single PUFA enzyme complex of three subunits each with multiple catalytic domains for the biosynthesis. Detailed analysis of genes in the aerobic pathway showed a key gene involved in the introduction of the first double bond into stearic acid, synthesized by Type I FAS, is missing from the genome, indicating this pathway is incomplete. This observation was recently confirmed by our *in vivo* feeding experiment. Co-existence of both aerobic and anaerobic pathways had been observed in *Thraustochytrids* [121, 122]. In *Schizochytrium sp.*, a Δ12 desaturase activity is missing from the aerobic pathway, while in *Thraustochytrium aureum*, the aerobic pathway is intact. Similar to *Thraustochytrium* sp. 26185, the anaerobic pathway is believed to be primarily responsible for the biosynthesis of VLCPUFA in these two species. It is likely that the aerobic pathway is a progenitor system, while the anaerobic pathway is newly acquired, and after acquisition of this efficient system, the progenitor pathway becomes relic in the species. During evolution, different components in the aerobic pathway can be lost or retained in different species.

Genomic analysis of genes involved in the assembly of glycerolipids indicated that freshly-synthesized VLCPUFA would be effectively incorporated into various phospholipids through both CDP-DAG pathway and head-activating pathway, as all the genes were found in the genome (Figure 3-5). In addition, the flux of freshly-synthesized fatty acids can go through several

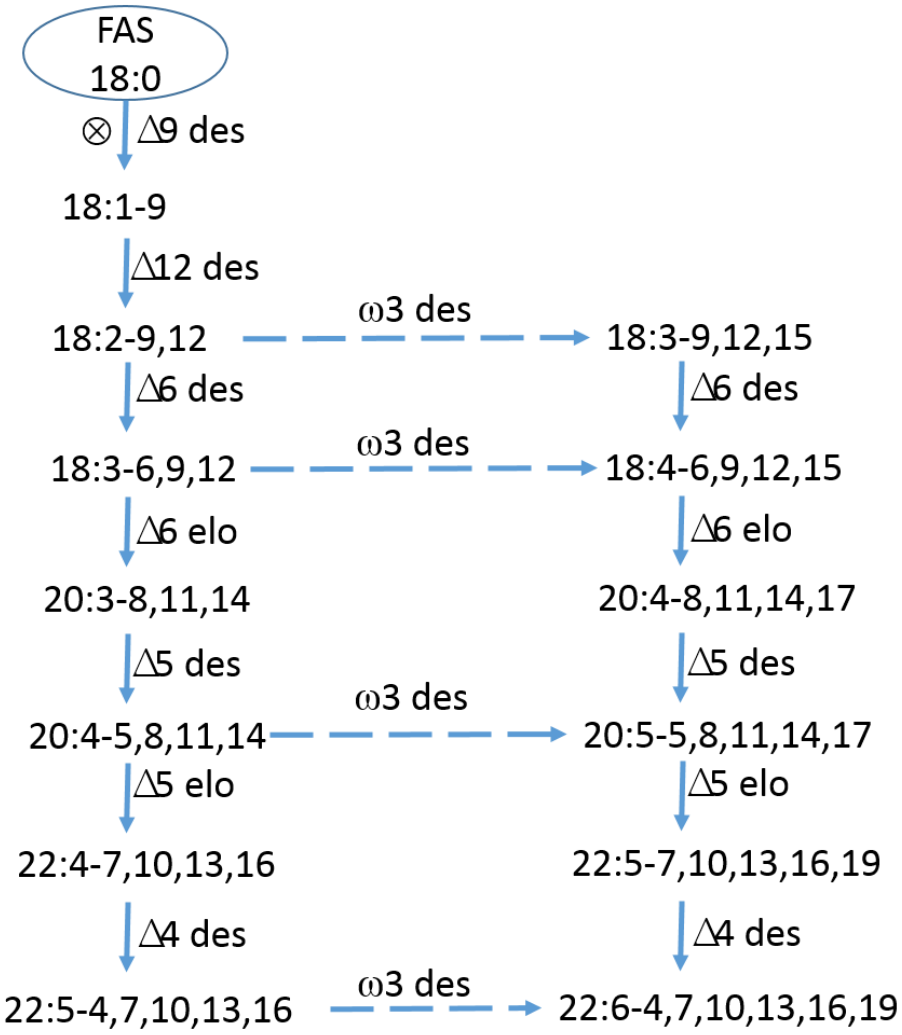

Figure 3-4. The aerobic pathway for the VLCPUFA biosynthesis in *Thraustochytrium* sp. 26185. "⊗" mark indicates the missing gene corresponding to the metabolic step in the species.

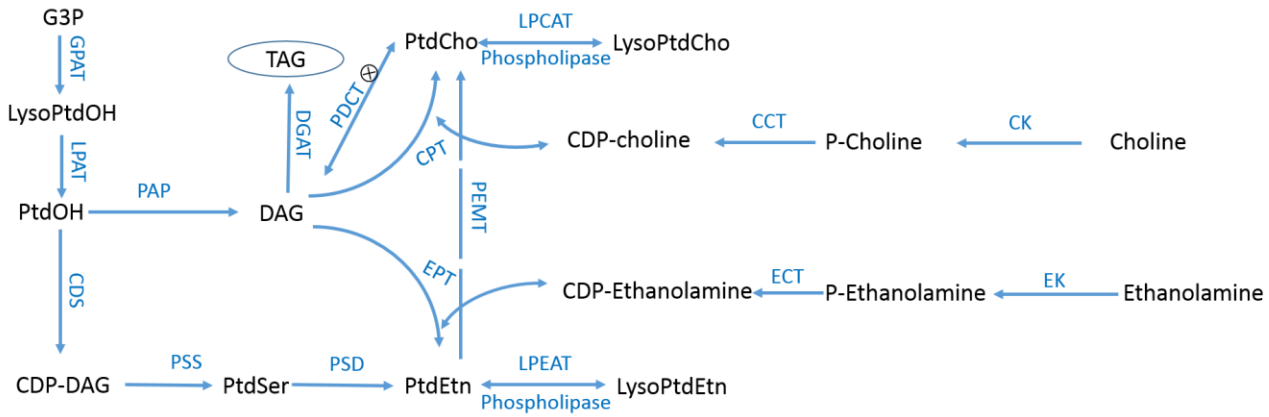

Figure 3-5. The pathway for the acyl trafficking among glycerolipids in *Thraustochytrium* sp. 26185.

"⊗" mark indicates the missing gene corresponding to the metabolic step in the species.

pathways into storage glycerolipids. They can be esterified onto the *sn*-1 and *sn*-2 positions of PC through acyl-editing or be used to acylate G3P for the *de novo* synthesis of DAG or be used for *sn*-3 acylation of DAG to generate TAG directly [92]. However, the relative flux efficiency of nascent VLCPUFA through these pathways is unknown in the *Thraustochytrium* sp. 26185. Acyl-editing of PC is believed to be important in the acyl trafficking in plants where LPCAT plays an important role to trafficking freshly-synthesized saturated and monounsaturated fatty acids from chloroplast to PC for further desaturation and other modification (hydroxylation and epoxidation for example) [56, 123]. In plants, *de novo* synthesized DAG starting from G3P is mainly used to synthesize PC while PC-derived DAG is the main precursor for the TAG synthesis generated either by PDCT or possibly reverse catalytic activity of CPT [92]. This mechanism indicates that fatty acids on TAG have already been modified on PC. Intriguingly, the candidate gene for phosphatidylcholine: diacylglycerol phosphocholine transferase (PDCT) to produce DAG from PC was not detected in the genome sequence of *Thraustochytrium* sp. 26185. This implies that, unlike plants, this species might not use PC-derived DAG generated by PDCT for the TAG biosynthesis. Instead, DAG precursor for the TAG biosynthesis might be either the *de novo* one from the Kennedy pathway or PC-derived one generated by reverse activity of CPT, although the latter activity has not been absolutely confirmed in any species.

3.6 Acknowledgments

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3.7 Author contribution

Xiao Qiu was responsible for the overall supervision of the project. Xianming Zhao, Dauenpen Meesapyodsuk, Cunmin Qu and Xiao Qiu contributed in discussion of the hypotheses, laying the objectives. Xianming Zhao and Xiao Qiu designed of experiments. Xianming Zhao performed the experiments and wrote the original manuscript. Xiao Qiu revised the manuscript.

4. Analysis of the biosynthetic process of fatty acids in *Thraustochytrium*[2]

In chapter 3, it was concluded from the genomic gene survey that *Thraustochytrium* sp. 26185 might possess both the aerobic and anaerobic pathways for VLCPUFA biosynthesis. However, which pathway is more important for VLCPUFA biosynthesis in this species remains to be determined, although there was evidence that the anaerobic pathway might be mainly responsible for the biosynthesis. In this chapter, several pieces of evidence confirmed that *Thraustochytrium* sp. 26185 mainly uses a PUFA synthase for VLCPUFA biosynthesis. In addition, it was found that odd-chain saturated fatty acids are synthesized by type I FAS using propionic acid as the initial precursor.

4.1 Abstract

Thraustochytrium sp. 26185 is a marine protist producing a specific profile of nutritionally-important fatty acids, including very long chain polyunsaturated fatty acids (VLCPUFAs) docosahexaenoic acid (DHA, 22:6n-3), even-chain saturated fatty acids (SFAs) palmitic acid (16:0), and odd-chain SFAs pentadecanoic acid (15:0). To study how these fatty acids are synthesized, a series of radiolabeled precursors were used to trace the biosynthetic process *in vivo* and *in vitro*. When *Thraustochytrium* sp. 26185 was fed with long chain fatty acid intermediates such as [1-^{14}C]-oleic acid, [1-^{14}C]-linoleic acid and [1-^{14}C]-α-linolenic acid, no VLCPUFAs were produced, indicating that the aerobic pathway for the biosynthesis of VLCPUFAs was not functional in *Thraustochytrium* sp. 26185. When fed with [1-^{14}C]-acetic acid, both SFAs and VLCPUFAs were labeled, and when fed with [1-^{14}C]-propionic acid, mainly SFAs were labeled. However, when fed with [1-^{14}C]-acetic acid in the presence of cerulenin, a type I FAS inhibitor, only VLCPUFAs were labeled, and when fed with [1-^{14}C]-propionic acid in the presence of cerulenin, neither SFAs nor VLCPUFAs were labeled. This result clearly indicates that the type I fatty acid synthase (FAS) in *Thraustochytrium* sp. 26185 could use acetic acid and propionic acid as the primers to synthesize even-chain and odd-chain SFAs, respectively, and VLCPUFAs were synthesized by the PUFA

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synthase using acetic acid as the primer. In addition, radioactive acetic acid could label both phospholipids (PL) and triacylglycerols (TAG), and VLCPUFAs appeared first and were largely accumulated in PL, whereas TAG accumulated much more SFAs than VLCPUFAs. The *in vitro* assay with [1-^{14}C]-malonyl-CoA in presence of cerulenin showed that the crude protein of *Thraustochytrium* sp. 26185 produced only VLCPUFAs, not SFAs, further confirming the role of the PUFA synthase in the biosynthesis of VLCPUFAs. Collectively, these results have elucidated the biochemical mechanisms for the biosynthesis of all fatty acids in *Thraustochytrium* sp. 26185.

4.2 Introduction

Thraustochytrium sp. 26185 is a marine protist that produces two groups of special fatty acids, very long chain polyunsaturated fatty acids (VLCPUFAs) such as docosahexaenoic acid (DHA; 22:6n-3) and docosapentaenoic acid (n-6 DPA; 22:5n-6), and odd-chain saturated fatty acids (SFAs) such as 15:0. It is well known that even-chain saturated fatty acids are usually synthesized by either type I or type II fatty acid synthase (FAS) using acetate as a primer and malonyl-ACP (acyl carrier protein) as an extender. Type I FAS is usually a single polypeptide mega-enzyme with multiple catalytic functions mainly found in animals and fungi, where each biochemical reaction is catalyzed by a distinct domain [124]. Type II FAS consists of several mono-functional enzymes and is mainly found in plants, bacteria and archaea where each biochemical reaction is catalyzed by a discrete enzyme [125]. Unlike the availability of much information on the biosynthesis of even-chain SFAs, the biosynthesis of odd-chain SFAs is less studied, because of their rare occurrence in nature, less evidence for their nutritional value and fewer tools available for the research. Nevertheless, the biosynthesis of odd-chain SFAs has been studied in many species, showing they are mainly synthesized by the same FAS system with a different primer. In the 1960s, Horning et al. reported that enzyme extracts from the epididymal adipose tissue of rat were able to synthesize 15:0 and 17:0 using propionyl-CoA as a precursor [126, 127]. At that time, it was believed that this propionyl-CoA-incorporating activity was characteristic of enzymes from some specific living species such as rat without general implication due to the rare occurrence of odd-chain fatty acids in other species. However, with the increasing findings of odd-numbered straight chain saturated and unsaturated fatty acids in many species, including bacteria, fungi, algae and plants [128-130], the biosynthesis of these fatty acids has drawn more scientific attention.

There are two distinct pathways for the biosynthesis of VLCPUFAs [43]. The aerobic pathway involves alternating desaturation and elongation and is mainly found in animals and eukaryotic microorganisms. In the aerobic pathway, synthesis of VLCPUFAs starts with introducing double bonds to intermediate long chain saturated fatty acids synthesized by FAS such as stearic acid (18:0). The last step of the biosynthesis of DHA, for instance, in microorganisms, involves a Δ4 desaturase to introduce the final double bond [73], whereas the last step of the DHA biosynthesis in mammals involves a two-carbon shortening of 24:6n-3 through one cycle of β-oxidation [131]. It is noteworthy that humans lack the Δ12 and Δ15 desaturases for introducing the two double bonds to convert oleic acid into linoleic acid and α-linolenic acid [132, 133], respectively, and thus humans cannot synthesize VLCPUFAs *de novo*. On the other hand, the anaerobic pathway utilizes a polyketide synthase-like mega-enzyme called PUFA synthase to synthesize VLCPUFAs directly from acetate precursor [47]. Double bonds are introduced during chain elongation processes [43], similar to the way *Escherichia coli* synthesizes unsaturated fatty acids [134].

Our earlier research identified the first Δ4 desaturase in *Thraustochytrium* sp. 26185, indicating the presence of the aerobic pathway for VLCPUFA biosynthesis in the species [73]. However, our recent work revealed a highly-active PUFA synthase in the anaerobic pathway as well as various desaturases and elongases in the aerobic pathway for the biosynthesis of VLCPUFAs in *Thraustochytrium* sp. 26185 [135, 136]. The goal of the present study is to further clarify the biosynthetic process of the fatty acids in the species, particularly for VLCPUFAs and odd-chain fatty acids, using a series of radiolabeled precursors with the view to elucidate the biochemical mechanisms for the biosynthesis of all fatty acids in *Thraustochytrium* sp. 26185.

4.3 Materials and methods

4.3.1 Culturing of *Thraustochytrium* sp. 26185

Thraustochytrium sp. 26185 was purchased from the American Type Culture Collection and cultured in GY medium consisting of 1% (w/v) yeast extract, 3% (w/v) D-glucose, and 1.75% (w/v) artificial sea salts (Sigma-Aldrich, St. Louis, MO, USA) at 25°C.

4.3.2 Lipid separation

Total lipids were extracted following a modified Bligh & Dyer method [137] using chloroform and methanol. One mL of radiolabeled cell culture was quenched by 1 mL of 85^{o}C isopropanol for 10

min before adding 3 mL of methanol, 2 mL of chloroform and 0.6 mL of water. The sample was mixed vigorously before centrifugation at 2,200 rpm for 10 min. The lower chloroform phase was collected, and the upper aqueous phase was back-extracted with 2 mL of chloroform. The combined chloroform phase was dried under nitrogen gas at around 35^{o}C. Total lipids were separated into different lipid classes including triacylglycerol (TAG), free fatty acids, diacylglycerol (DAG) and polar lipids (mainly phospholipids, PL) by thin layer chromatography (TLC). Briefly, each sample was carefully loaded onto a glass-backed silica gel plate (Analtech, uniplate, 20×20cm, Newark, DE, USA) and the plate was developed in a solution consisting of hexane/diethyl ether/acetic acid (70/30/1, v/v/v). Radioactivity on the TLC plate was detected using a storage phosphor screen (Amersham Biosciences, Buckinghamshier, UK) in an exposure cassette for at least 4 h. The screen was visualized with a Typhoon FLA 7000 scanner (GE Healthcare Life Sciences, Malborough, MA, USA) equipped with an IP filter under the phosphor image scanning mode.

4.3.3 Fatty acid analysis

Thraustochytrium sp. 26185 cells were harvested by centrifugation at 4,000 rpm for 5 min after 24 h of culturing when the OD_{600} reached 0.5. Total fatty acids were converted into fatty acid methyl esters (FAMEs) by heating the cell pellet in 2 mL of 2% sulfuric acid in methanol at 85^{o}C for 1 h. After transmethylation, the sample was cooled down to room temperature and 1 mL of 0.88% KCl and 2 mL of hexane was added. The mixture was then agitated vigorously and centrifuged at 2,200 rpm for 5 min for phase separation. The upper hexane phase containing FAMEs was collected and dried under nitrogen gas before being analyzed by an Agilent 7890A gas chromatography (Agilent, Santa Clara, CA, USA) equipped with a DB-23 column (30 m × 0.25 mm) with 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). FAMEs of TAG and PL on TLC plates were prepared by direct transmethylation of the scraped lipid bands by heating in 2 mL of 2% sulfuric acid in methanol at 85^{o}C for 1 h and extracted as described above. FAMEs of radiolabeled samples were separated into SFAs and VLCPUFAs by $AgNO_3$-TLC developed by hexane/diethyl ether/acetic acid (94/4/2, v/v/v). The plate was pre-sprayed with 20 mL of 10% $AgNO_3$ in acetonitrile and dried at 105^{o}C before loading samples. The radioactivity on the plate was exposed to a storage phosphor screen and visualized as described above.

4.3.4 Feeding of radiolabeled precursor and intermediate fatty acids

In order to trace the biosynthetic pathways of various fatty acids produced by *Thraustochytrium* sp. 26185, ^{14}C-labeled primers and intermediate fatty acids including 10 µCi of [1-^{14}C]-acetic acid, [1-^{14}C]-propionic acid, [1-^{14}C]-oleic acid, [1-^{14}C]-linoleic acid and [1-^{14}C]-α-linolenic acid (American Radiolabeled Chemicals, Inc. St. Louis, MO, USA) were fed to *Thraustochytrium* sp. 26185 at the log phase. At each time point of feeding (1, 2, 5, 10, 30 and 60 min for acetic acid and propionic acid, 60 min for others), total lipids were extracted and separated on a TLC plate into TAG, DAG and PL. Each lipid class was eluted from the TLC plate and used for either radioactivity counting or FAME preparation. Feeding of [1-^{14}C]-acetic acid and [1-^{14}C]-propionic acid in the presence of cerulenin was also conducted according to a previously reported method [138]. Briefly, 1 mL of *Thraustochytrium* sp. 26185 growing in log phase was harvested by centrifugation and resuspended in 1 mL of fresh medium. 50 µM of cerulenin was added 30 min before adding 10 µM of either [1-^{14}C]-acetic acid or [1-^{14}C]-propionic acid. After labeling for 60 min, total lipids were extracted from cell pellet, and then the FAMEs were prepared, separated into SFAs and PUFAs on a TLC plate and visualized as described above.

Lipid classes were eluted from the TLC plate by the following procedure. Briefly, the scraped silica gel containing each lipid class was extracted twice by 2.5 mL of chloroform/methanol/water (5/5/1, v/v/v). After a brief centrifugation, the combined liquid phase was then combined with 2 mL of chloroform, and 1.5 mL of 0.88% KCl for phase separation by centrifugation at 2,200 rpm for 10 min. The lower chloroform phase was collected. The sample was extracted again by 2 mL of chloroform. The combined chloroform was then dried under nitrogen gas. FAMEs were also eluted from TLC plates for radioactivity counting. Briefly, FAMEs on scraped silica gel were extracted by 2 mL of hexane/isopropanol/water (60/40/5, v/v/v). After adding 1 mL of 1M KCl to precipitate Ag^+, the mixture was centrifuged at 2,200 rpm for 10 min. The upper hexane was collected, and the sample was extracted again with 1 mL of hexane. The collected hexane was dried by nitrogen gas.

For quantitative analysis of radioactivity, one part of the eluted lipid classes or the FAMEs extracted from $AgNO_3$-TLC plates were counted by a Tri-Carb 2910 TR liquid scintillation analyzer (Perkin Elmer, Waltham, MA, USA). The radioactivity was presented in the form of disintegrations per minute (DPM).

4.3.5 *In vitro* assays

The crude protein of *Thraustochytrium* sp. 26185 was prepared by vigorous agitation of 1 mL of cell culture ($OD_{600} \approx 1$) with 0.5 mL of 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA) using a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK, USA). The mixture was agitated for 3 min with a 30 second ice incubation every 1 min of agitation. Afterwards, the mixture was centrifuged at 12,000 rpm at 4°C for 5 min and the supernatant was collected for *in vitro* enzymatic assays. Crude protein of *Thraustochytrium* sp. 26185 lysate was incubated at 25°C for 1 h with the presence of 100 µM acetyl-CoA, 500 µM malonyl-CoA (a mixture of 0.2 µCi, 20 µM [1-^{14}C]-malonyl-CoA and unlabeled malonyl-CoA), 1 mM NADP and 1 mM NADPH in a reaction mixture of totally 200 µl. Twenty µM cerulenin (Sigma-Aldrich, St. Louis, MO, USA) was used to inhibit the activity of type I FAS [139].

4.4 Results

4.4.1 Fatty acid profile of *Thraustochytrium* sp. 26185

To facilitate the investigation of fatty acid biosynthesis in *Thraustochytrium* sp. 26185, the fatty acid composition of cells grown for 24 h prior to the addition of radiolabeled precursors was analyzed. As shown in Figure 4-1, two distinct groups of fatty acids, SFAs and VLCPUFAs, were observed in *Thraustochytrium* sp. 26185. For VLCPUFAs, DHA (22:6n-3) was the most abundant, followed by DPA (22:5n-6). Eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6) were minor components, together accounting for approximately only 2% of the total fatty acids (Table 4-1). For saturated fatty acids, palmitic acid (16:0) was the most abundant, followed by 15:0, 14:0 and 17:0. 18:0 and 13:0 were minor saturated fatty acids. It is notable that *Thraustochytrium* sp. 26185 produced substantial amounts of odd-chain SFAs such as 15:0 and 17:0, together accounting for approximately 27% of the total fatty acids. Furthermore, oleic acid (18:1n-9), linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), three common 18 carbon unsaturated fatty acid intermediates for VLCPUFAs in the aerobic pathway, were not detected under the growth condition used.

4.4.2 Feeding *Thraustochytrium* sp. 26185 with radiolabeled long chain fatty acids

Our previous research showed that *Thraustochytrium* sp. 26185 possessed several genes corresponding to enzymes in the aerobic pathway for the biosynthesis of VLCPUFAs with possibly

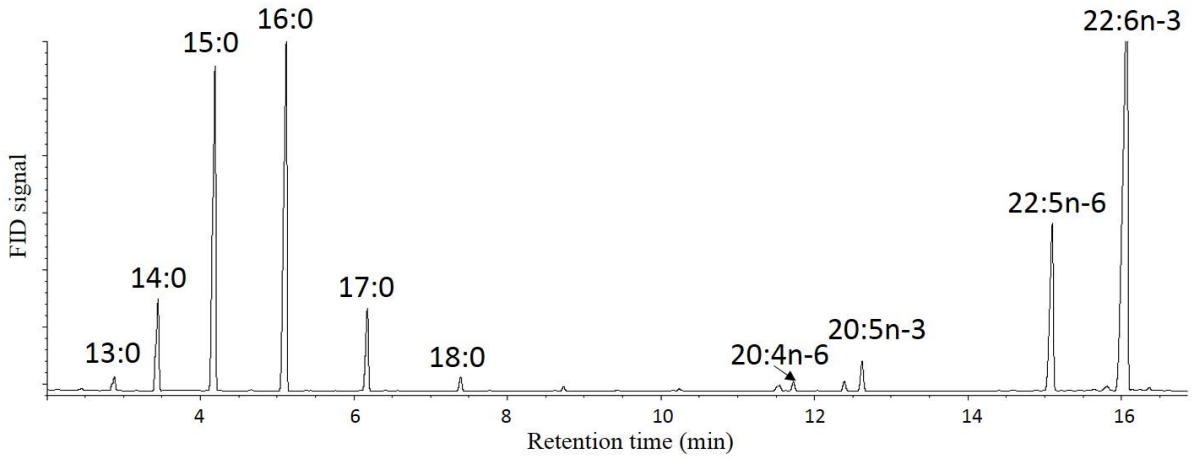

Figure 4-1. The representative fatty acid profile of *Thraustochytrium* sp. 26185.

Table 4-1. Fatty acid composition of *Thraustochytrium* sp. 26185.

Fatty acids	% of total fatty acids (Mean± SD)
13:0	0.73 ± 0.11a
14:0	4.27 ± 0.08b
15:0	21.60 ± 0.42c
16:0	21.82 ± 0.70c
17:0	5.76 ± 0.21d
18:0	0.90 ± 0.03a
20:4n-6 (ARA)	0.47 ± 0.01a
20:5n-3 (EPA)	1.61 ± 0.01e
22:5n-6 (DPA)	9.54 ± 0.16f
22:6n-3 (DHA)	32.11 ± 0.92g
Others	1.19 ± 0.44ae

Note: Values were reported as the means ± standard deviations from three biological replicates. Statistical analysis was done by IBM SPSS Statistics (Version 24) using the One-Way ANOVA Duncan's multiple range test. The means with the same letters are not statistically significantly different, while the means with different letters are statistically significantly different ($P<0.05$, $n=3$).

missing a few desaturation steps [135]. To prove if the aerobic pathway was functional for the biosynthesis of VLCPUFAs, three radiolabeled long-chain intermediate fatty acids, [1-^{14}C]-oleic acid, [1-^{14}C]-linoleic acid and [1-^{14}C]-α-linolenic acid, were used to trace the biosynthetic process in the aerobic pathway. As shown in Figure 4-2A, all ^{14}C-labeled fatty acids fed to *Thraustochytrium* sp. 26185 were readily taken up by *Thraustochytrium* sp. 26185 and incorporated into glycerolipids where the radioactivity appeared mostly in triacylglycerol (TAG) and polar lipid (mainly phospholipids, PL) fractions with little in DAG. To interrogate if any modification such as desaturation and elongation of the fed fatty acids in the aerobic pathway occurred, the fatty acid composition of the total lipids extracted from fed *Thraustochytrium* sp. 26185 was resolved by $AgNO_3$-TLC. As shown in Figure 4-2B, fed intermediate fatty acids were not utilized to synthesize VLCPUFAs through alternating desaturation and elongation, indicating the aerobic pathway for VLCPUFA biosynthesis in *Thraustochytrium* sp. 26185 was not functional.

4.4.3 Feeding *Thraustochytrium* sp. 26185 with radiolabeled acetic acid

Acetic acid is an initial precursor for the biosynthesis of all fatty acids including SFAs and VLCPUFAs. After synthesizing, both SFAs and VLCPUFAs would be incorporated into glycerolipids such as TAG, DAG and PL. As shown in Figure 4-3, in a one-hour time course of acetate labeling with six time points, the radioactivity of incorporated fatty acids was higher in PL than in TAG at initial few time points. With the labeling going on, the accumulation of radioactivity increased drastically in both PL and TAG fractions; however, labelled activity in TAGs increased more rapidly at later time points and eventually surpassed the radioactivity in PL in 60 minutes. Among the three labeled glycerolipids, DAG accumulated much less radioactivity relative to TAG and PL.

To investigate the composition of fatty acids in different lipid classes synthesized from the tracer, two abundant lipid classes TAG and PL were scraped from the TLC plate and their fatty acid profiles were resolved by $AgNO_3$-TLC. As shown in Figure 4-4, in one minute of feeding, VLCPUFAs were hardly detected while SFAs were obviously detected in TAG fraction. On the other hand, at the same time point, both SFAs and VLCPUFAs were detected at similar levels in PL fraction. With the labeling time going on, labeled SFAs and VLCPUFAs in both TAG and PL increased. From the time point of 30 minutes forwards, the amount of labeled VLCPUFAs remained significantly higher than that of SFAs in PL, whereas the amount of labeled SFAs was consistently higher than that of labeled VLCPUFAs in TAG throughout the time course. This result

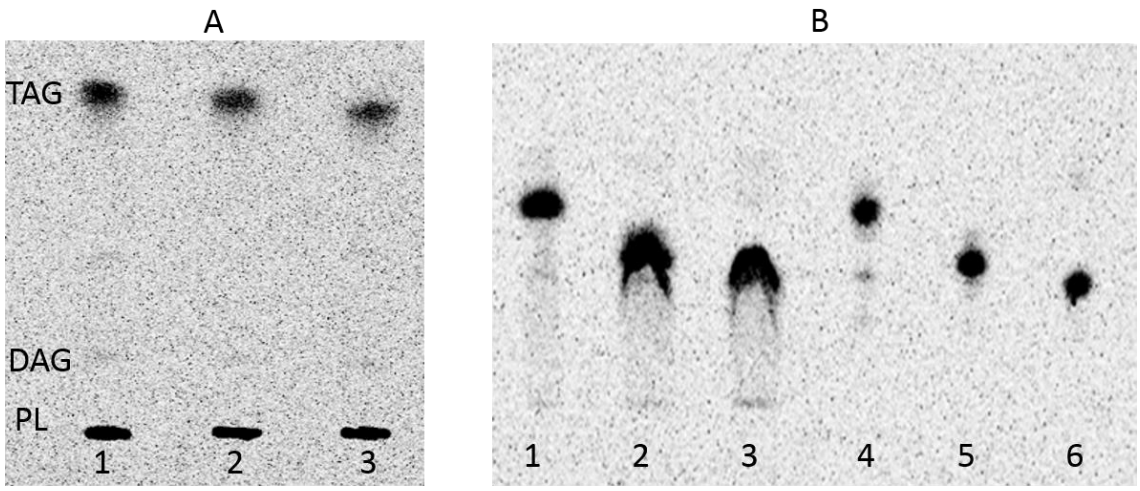

Figure 4-2. Analysis of total lipids and fatty acids from *Thraustochytrium* sp. 26185 fed with radiolabeled oleic, linoleic and linolenic acid.

A, TLC separation of total lipids developed by hexane/diethyl ether/acetic acid (70/30/1, v/v/v); B, $AgNO_3$-TLC separation of FAMEs of the total lipids developed by hexane/diethyl ether/acetic acid (94/4/2, v/v/v). Lane 1, [1-^{14}C]-oleic acid feeding; Lane 2, [1-^{14}C]-linoleic acid feeding; Lane 3, [1-^{14}C]-α-linolenic acid feeding; Lane 4, [1-^{14}C]-oleic acid methyl ester standard; Lane 5, [1-^{14}C]-linoleic acid methyl ester standard; Lane 6, [1-^{14}C]-α-linolenic acid methyl ester standard.

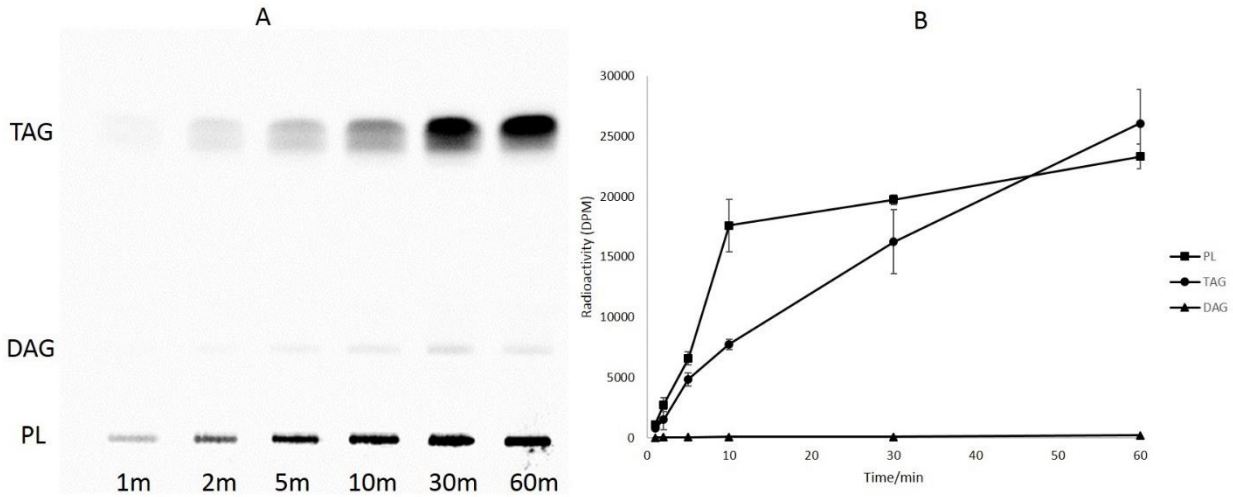

Figure 4-3.9 Analysis of total lipids from *Thraustochytrium* sp. 26185 fed with [1-^{14}C]-acetic acid for 1 h.

A, TLC separation of total lipids developed by hexane/diethyl ether/acetic acid (70/30/1, v/v/v). B, the amount of radioactivity in different lipid classes. Values were reported as the means ± standard deviations from three biological replicates.

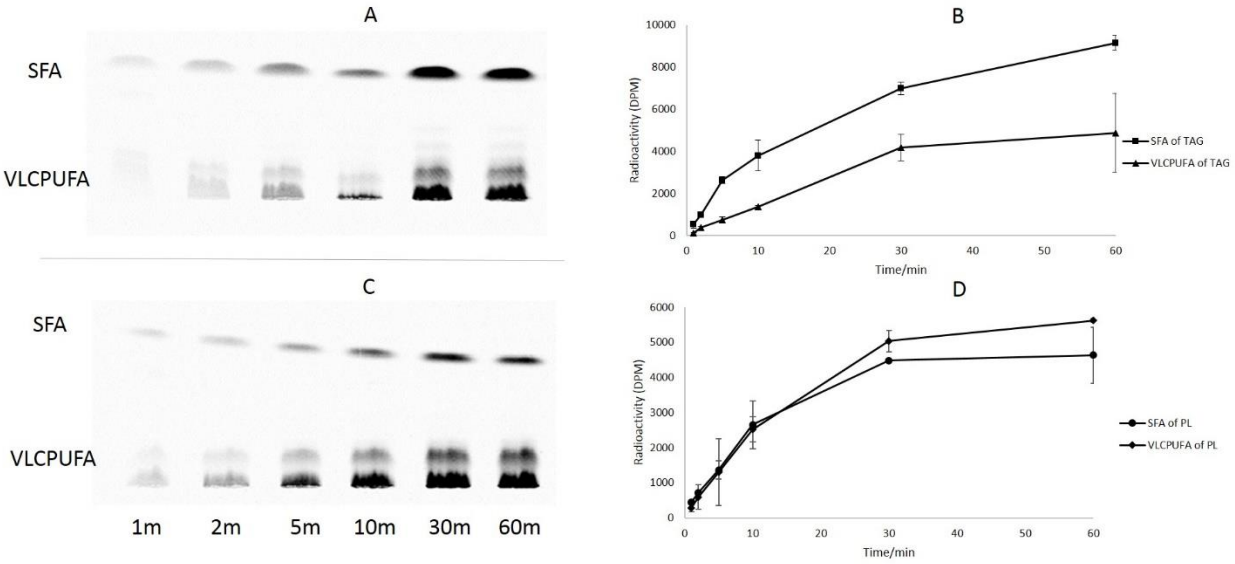

Figure 4-4. Analysis of fatty acid profile in TAG and PL from *Thraustochytrium* sp. 26185 fed with [1-^{14}C]-acetic acid for 1 h.

A, $AgNO_3$-TLC separation of FAMEs of TAG developed by hexane/diethyl ether/acetic acid (94/4/2, v/v/v). B, the amount of radioactivity in FAMEs of TAG. C, $AgNO_3$-TLC separation of FAMEs of PL developed by hexane/diethyl ether/acetic acid (94/4/2, v/v/v). D, the amount of radioactivity in FAMEs of PL. Values were reported as the means ± standard deviations from three biological replicates. At each time point of comparison of labelled SFA and VLCPUFA, * indicates significance at $P<0.05$ and ** indicates significance at $P<0.01$ differences.

indicates that SFAs are more favorably retained in TAG while VLCPUFAs are more preferentially incorporated in PL within one hour of labeling.

4.4.4 Feeding *Thraustochytrium* sp. 26185 with radiolabeled propionic acid in the presence of cerulenin

A significant feature of the fatty acid profile of *Thraustochytrium* sp. 26185 is the presence of a substantial amount of odd-chain saturated fatty acids. It is well known that even-chain fatty acids are synthesized by FAS using acetic acid as a primer and malonyl-ACP as an extender. It is thus assumed that odd-chain fatty acids in *Thraustochytrium* sp. 26185 might be synthesized by the same enzyme using propionic acid as a primer, as seen in rat [126, 127]. To test this hypothesis, radiolabeled propionic acid was used to trace the fatty acid biosynthesis in the presence or absence of cerulenin, an effective inhibitor for type I FAS, but not for PUFA synthase [139] when an appropriate dose was used. As shown in Figure 4-5, in the absence of cerulenin, unlike [1-^{14}C]-acetic acid feeding where both SFAs and VLCPUFAs were labeled, [1-^{14}C]-propionic acid feeding mainly labeled SFAs, not VLCPUFAs. Although the identity of these SFAs was not determined, we believe they are probably mainly odd-chain SFAs such as 15:0 and 17:0 as shown in Figure 4-1. However, in the presence of cerulenin, when *Thraustochytrium* sp. 26185 was fed with [1-^{14}C]-acetic acid, the radioactivity of SFAs was reduced by 92% while the radioactivity of VLCPUFAs was reduced by only 23%; and when *Thraustochytrium* sp. 26185 was fed with [1-^{14}C]-propionic acid, the radioactivity of SFAs was reduced by 96% while radiolabeled VLCPUFAs were barely detectable. This result indicates that the type I FAS in *Thraustochytrium* sp. 26185 is responsible for the synthesis of both even-chain and odd-chain fatty acids by utilizing acetic acid and propionic acid as the initial primers, respectively, and the PUFA synthase is solely responsible for the biosynthesis of VLCPUFAs by using acetic acid as the initial primer.

4.4.5 *In vitro* assay with radiolabeled malonyl-CoA

To further confirm that SFAs and VLCPUFAs were synthesized by type I FAS and PUFA synthase, respectively, the cell lysate of *Thraustochytrium* sp. 26185 was used for *in vitro* assays in the presence or absence of cerulenin [139]. As shown in Figure 4-6, in the absence of cerulenin, the crude enzymes were able to synthesize both SFAs and VLCPUFAs with acetyl-CoA and ^{14}C-malonyl-CoA. However, in the presence of cerulenin, the crude enzymes could synthesize only VLCPUFAs, but not SFAs. This result confirms that the biosynthesis of VLCPUFAs in

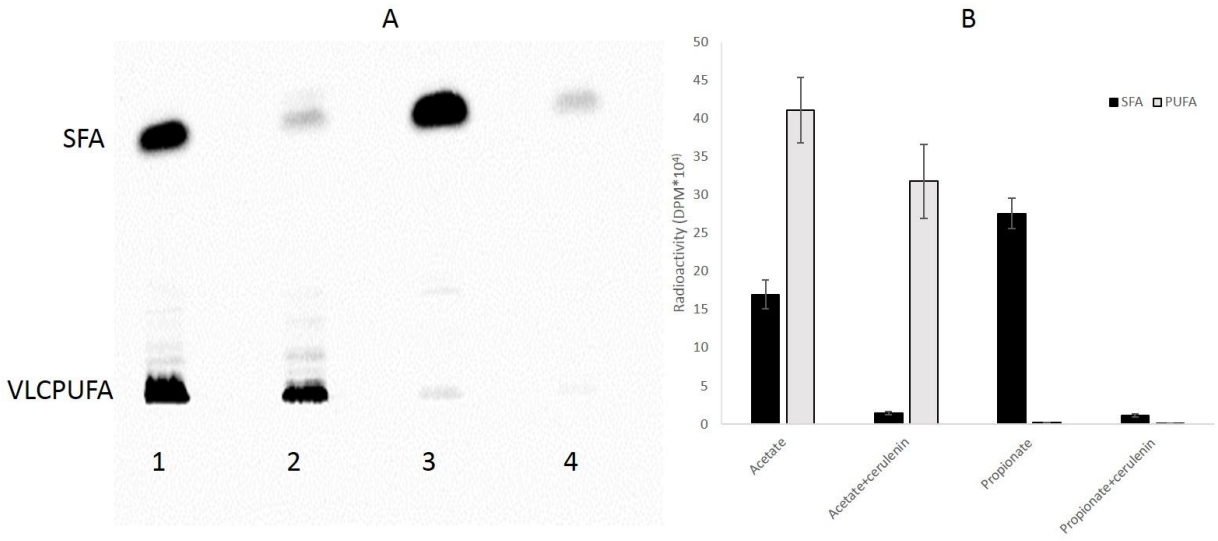

Figure 4-5. Analysis of fatty acid profile of total lipids from *Thraustochytrium* sp. 26185 fed with [1-^{14}C]-acetic acid and [1-^{14}C]-propionic acid in the presence/absence of cerulenin.
A, $AgNO_3$-TLC separation of the FAMEs developed by hexane/diethyl ether/acetic acid (94/4/2, v/v/v). B, the amount of radioactivity in the SFAs and VLCPUFAs. Lane 1 and 3 are [1-^{14}C]-acetic acid [1-^{14}C]-propionic acid labelings, respectively, in the absence of cerulenin. Lane 2 and 4 are [1-^{14}C]-acetic acid [1-^{14}C]-propionic acid labelings, respectively, in the presence of cerulenin. Values were reported as the means ± standard deviations from three biological replicates. Bars with the same letters are not statistically significantly different, while bars with different letters are statistically significantly different ($P<0.05$, $n=3$).

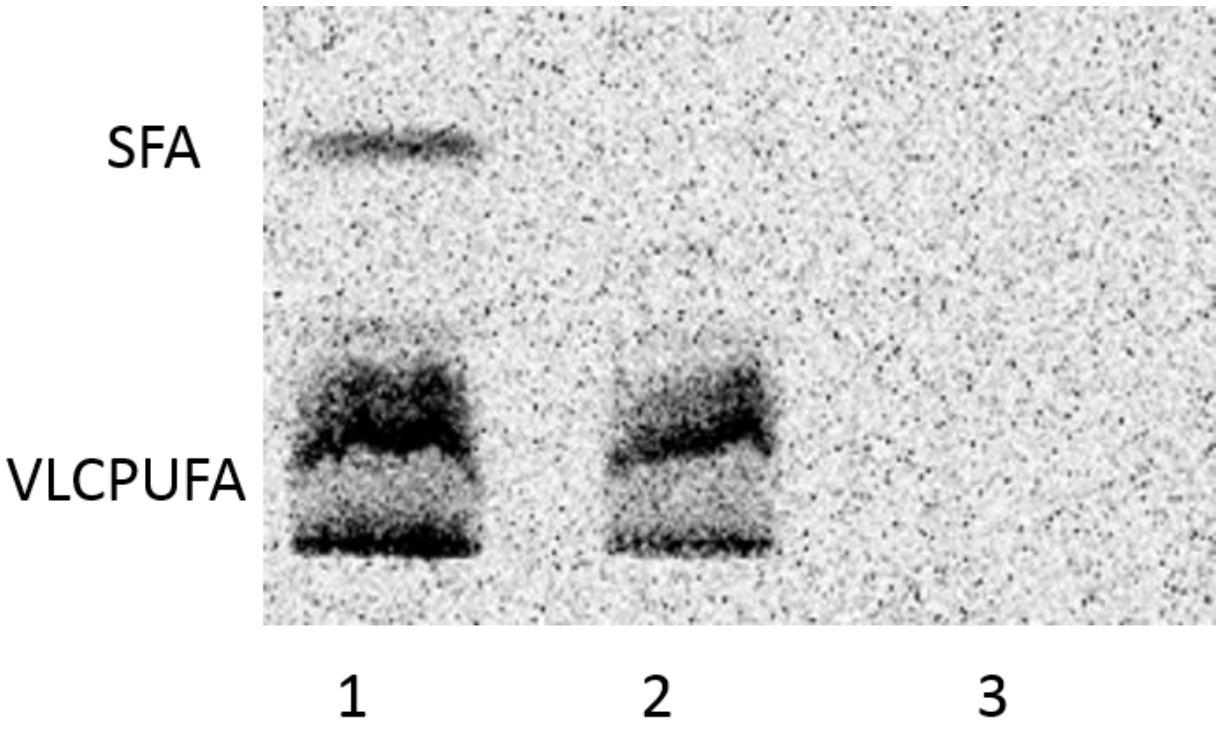

Figure 4-6. The representative $AgNO_3$-TLC of FAMEs prepared from *in vitro* assay.
Lane 1, crude protein with 100 µM acetyl-CoA, 500 µM malonyl-CoA (a mixture of [1-^{14}C]-malonyl-CoA and unlabeled malonyl-CoA), 1 mM NADP and 1 mM NADPH; Lane 2, the same as in Lane 1 with the addition of 20 µM cerulenin; Lane 3, the same as in Lane 1 with heat deactivated proteins.

Thraustochytrium sp. 26185, under the experimental conditions, is a *de novo* biosynthetic process catalyzed by the PUFA synthase, which is independent of long chain SFAs synthesized by type I FAS.

4.5 Discussion

Thraustochytrium is a genus of *Thraustochytriidae* family, a group of marine protists that are known for producing high amounts of VLCPUFAs [140]. Some of the species in the genus have been utilized for commercial production of DHA, a fatty acid of nutritional importance to human health. The species used in our study has a very specific fatty acid profile consisting of two distinct groups of fatty acids, VLCPUFAs and SFAs. The VLCPUFAs in this species are mainly DHA and DPA, whereas the SFAs are mainly 16:0, 15:0 and 17:0. Intermediate fatty acids for the biosynthesis of DHA and DPA in the aerobic pathway such as oleic acid, linoleic acid and α-linolenic acid are barely detectable under the growth condition. This specific fatty acid profile raises a question of how these fatty acids are synthesized in *Thraustochytrium* sp. 26185.

Our recent genome sequencing of the species identified two fatty acid synthesizing enzymes, type I FAS and PUFA synthase in *Thraustochytrium* sp. 26185. It is well known that even-chain SFAs are synthesized by type I FAS using acetyl-CoA as a primer and malonyl-ACP as an extender in fungi and animals. In this study, radiolabeled propionic acid was used to investigate whether or not the same FAS is also responsible for the biosynthesis of odd-chain fatty acids using a different primer in *Thraustochytrium* sp. 26185. When fed with propionic acid in the absence of cerulenin, a specific inhibitor of type I FAS, *Thraustochytrium* sp. 26185 produced mainly SFAs, not VLCPUFAs. When fed with propionic acid in the presence of cerulenin, no SFA was produced, indicating odd-chain saturated fatty acids such as 15:0 and 17:0 are indeed synthesized by type I FAS using propionic acid as the primer in *Thraustochytrium* sp. 26185.

There are two distinct pathways for the biosynthesis of VLCPUFAs. The aerobic pathway involves alternative elongation and desaturation of intermediate long chain fatty acids. However, those intermediate fatty acids such as oleic, linoleic and α-linolenic acid, were rarely seen in the fatty acid profile of *Thraustochytrium* sp. 26185 (Figure 4-1), implying that *Thraustochytrium* sp. 26185 lacks the ability to introduce initial and methyl-end double bonds to long chain SFAs, despite it possessing the ability to introduce carboxyl-end double bonds by front-end desaturases [73]. In addition, feeding radiolabeled intermediate fatty acids, such as oleic acid, linoleic acid and α-

linolenic acid resulted in those fatty acids going directly into glycerolipids with no further modification (elongation or desaturation). These results confirm that the aerobic pathway was clearly not functional for the biosynthesis of DHA and DPA in *Thraustochytrium* sp. 26185. On the other hand, *in vivo* assays with radiolabeled acetate in the presence or absence of cerulenin showed VLCPUFAs were actively synthesized from the feeding precursor under both conditions. In addition, *in vitro* assays with [1-^{14}C]-malonyl-CoA revealed that VLCPUFAs were produced in the presence of cerulenin. Collectively, these results unambiguously demonstrate that the PUFA synthase is solely responsible for the biosynthesis of VLCPUFAs in *Thraustochytrium* sp. 26185. The anaerobic pathway is a *de novo* VLCPUFA biosynthetic process firstly discovered in *Schizochytrium*, a closely related genus with *Thraustochytrium* sp. 26185, and later found in many other marine microorganisms [47, 91, 122, 141, 142]. It is efficient for the biosynthesis of VLCPUFAs probably due to fewer catalytic steps, and thus produces cleaner fatty acid profiles, which are advantageous over the aerobic pathway for the production of VLCPUFAs for animal and human nutrition.

Production of VLCPUFAs in *Thraustochytrium* sp. 26185 involves not only the synthesis of the fatty acids, but also effective incorporation of these fatty acids to glycerolipids for storage. Feeding radiolabeled acetic acid showed that the incorporation behaviors of freshly synthesized SFAs and VLCPUFAs were very different. Throughout all feeding stages, SFAs were more efficiently accumulated in TAG, whereas VLCPUFAs are more preferably incorporated into PL. The initial incorporation efficiency of VLCPUFAs in PL was high, and with increased labeling time, the incorporation of VLCPUFAs in PL remains even faster than that in TAG. These results indicate that freshly synthesized VLCPUFAs are first incorporated into PL and then mobilized to TAG for storage. However, it is unknown how VLCPUFAs are trafficked from PL to TAG. In plants, unsaturated fatty acids are synthesized by the aerobic pathway where the desaturation occurs mainly on acyl chains of phospholipids, and thus freshly synthesized fatty acids from chloroplasts mainly go to phospholipids for editing. After that, phospholipids (mainly phosphatidylcholine, PC) are converted into DAG. This PC-derived DAG, other than *de novo* synthesized DAG, is the major precursor for the TAG synthesis [56, 91, 92, 123]. In other words, the incorporation of freshly synthesized fatty acids into PL and then to TAG for storage is an essential process because of the need of acyl editing on PC through the Land Cycle [63] in plants. In *Thraustochytrium* sp. 26185, however, fatty acids synthesized by either type I FAS or PUFA synthase are end products without

further modification for incorporation into TAG for storage. Then, it remains to be resolved that how important the shuffling process of VLCPUFAs from PL to TAG is for production of the fatty acids and what the shuffling mechanism of VLCPUFAs between PL and TAG is in *Thraustochytrium* sp. 26185. Further analysis of acyl trafficking among glycerolipids in the species will be required to answer these questions.

4.6 Acknowledgments

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4.7 Author contribution

Xiao Qiu was responsible for the overall supervision of the project. Xianming Zhao and Xiao Qiu designed the study. Xianming Zhao performed the experiments and wrote the original manuscript. Xiao Qiu revised the manuscript.

5. Very long chain polyunsaturated fatty acids accumulated in triacylglycerol are channeled from phosphatidylcholine in *Thraustochytrium*[3]

In chapter 4, we answered the question of how different fatty acids are synthesized in *Thraustochytrium* sp. 26185. This study was designed to address the question of how freshly-synthesized fatty acids are incorporated into different glycerolipids. We first investigated the glycerolipid profile of *Thraustochytrium* sp. 26185 using lipidomic tools, and then traced the acyl and backbone flux during glycerolipid assembly using radiolabeled tracers. A very different incorporation pattern was discovered between SFAs and VLCPUFAs. SFAs are readily incorporated in both TAG and PC after being synthesized. In contrast, VLCPUFAs are incorporated in PC first, and then channeled to TAG possibly via DAG as the intermediate.

5.1 Abstract

Thraustochytrium sp. 26185 is a marine protist that can accumulate a large amount of nutritionally important very long chain-polyunsaturated fatty acids (VLCPUFA) such as docosahexaenoic acid (DHA) in triacylglycerols (TAG). These fatty acids are primarily synthesized by a PUFA synthase; however, how these freshly synthesized VLCPUFAs are channeled into TAG remains unknown. In this study, the glycerolipid profile of *Thraustochytrium* sp. 26185 at log and stationary growth stages was first analyzed by lipidomic tools, and then ^{14}C-acetate and ^{14}C-glycerol were used to trace the flux of fatty acids and backbone in glycerolipids. Lipidomic analysis showed that VLCPUFAs were mostly allocated to phosphatidylcholine (PC) and TAG. PC possessed a relatively stable profile of VLCPUFAs at the two growth stages; whereas, TAG species with one or two VLCPUFAs were significantly increased at the stationary phase. Freshly-synthesized VLCPUFAs labeled by ^{14}C-acetate were predominately incorporated into PC initially, while at the late time point of labeling, these fatty acids were mostly found in TAG. Positional analysis showed

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that PC had either one VLCPUFA at its *sn*-2 position (PC1) or two VLCPUFAs at both *sn*-1 and *sn*-2 positions (PC2), while TAG incorporated these fatty acids almost exclusively at the *sn*-2 position with similar stereospecific structure as PC1. Similarly, ^{14}C-glycerol was more efficiently incorporated into PC1 than TAG initially, and at the late time point of labeling, it was mostly found in TAG, and DAG and PC1 shared a similar incorporation pattern. These results indicate that VLCPUFAs in TAG are mainly channeled from PC likely through diacylglycerol as the intermediate.

5.2 Introduction

Omega-3 very long chain polyunsaturated fatty acids (VLCPUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have drawn increasing attentions due to their potential roles in health promotion and disease prevention [143]. Currently, the major sources of such fatty acids are marine alga and fish oil. However, the *de novo* biosynthesis of VLCPUFAs occurs mainly in microorganisms such as algae, bacteria and fungi through two metabolic pathways [43]. The aerobic pathway goes through alternating elongations and desaturations to introduce double bonds and to extend the chain length of long chain fatty acids, while the anaerobic pathway utilizes a single multifunctional enzyme called PUFA synthase to synthesize VLCPUFAs from the initial precursor acetic acid. After synthesis, VLCPUFAs are mainly destined to two types of glycerolipids; phospholipids (PL) and triacylglycerols (TAG). Phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS) are membrane lipids functioning to maintain the integrity and dynamics of cellular membrane systems, while TAG is the main storage form of glycerolipids serving as carbon and energy reserves.

The biosynthetic pathways of PL and TAG have been elucidated using isotope tracers, a powerful technique probing the relationships of initial substrates and final products [55, 61, 144]. For the biosynthesis of a group of phospholipids such as PS, PG and PI, phosphatidic acid (PA) serves as the backbone precursor for the attachment of a head group through an intermediate cytidine diphosphate-diacylglycerol (CDP-DAG). For the biosynthesis of another group of phospholipids such as PC and PE, DAG serves as the backbone precursor for the attachment of a head group donated by CDP-choline or CDP-ethanolamine. For the biosynthesis of TAG, two fatty acids are sequentially acylated to glycerol-3-phosphate at *sn*-1 and *sn*-2 positions, giving PA, which is then

converted to DAG by removing the phosphate group at the *sn*-3 position. DAG is finally acylated with a third fatty acid at the *sn*-3 position to form TAG. As DAG is a central intermediate for both PC and TAG, efforts have recently been made to elucidate their relationship in plants [91, 92]. However, the exact relationship among DAG, PC and TAG is still not well understood, particularly for those marine microorganisms accumulating nutritionally important VLCPUFAs in TAG.

Thraustochytrium sp. 26185 is a marine protist that can produce a relatively large amount of VLCPUFAs, mostly DHA. Therefore, *Thraustochytrium* sp. 26185 and other related species have attracted much scientific and industrial interest in the production of DHA for commercial uses through fermentation and metabolic engineering [80, 145-147]. Our previous work indicates that DHA is solely-synthesized by the anaerobic pathway using a polyketide synthase-like PUFA synthase comprising three large subunits each with multiple functional domains; whereas, the aerobic pathway employing desaturases and elongases is incomplete in *Thraustochytrium* sp. 26185 [136, 148]. This study investigated how freshly synthesized VLCPUFAs were channeled into TAG for storage using pulse-chase and steady-state labeling of fatty acids and the glycerol backbone in *Thraustochytrium* sp. 26185. The results reveal that VLCPUFAs are mainly channeled to TAG from PC through DAG as the intermediate in *Thraustochytrium* sp. 26185. The finding of this unusual route of channeling VLCPUFAs to storage lipids not only contributes to the understanding of the acyl assembly during the biosynthesis of glycerolipids in these types of microorganisms, but also helps design strategies to improve the flux process for the heterologous production of these nutritionally important fatty acids in other eukaryotes.

5.3 Materials and Methods

5.3.1 Cultivation of *Thraustochytrium* sp. 26185

Thraustochytrium sp. 26185 was purchased from the American Type Culture Collection. It was maintained on BY^+ agar plates containing 0.1% yeast extract (w/v), 0.1% peptone (w/v) and 0.5% D-glucose in simulated sea water. It was cultured with shaking in GY medium consisting of 1% (w/v) yeast extract, 3% (w/v) D-glucose, and 1.75% (w/v) artificial sea salts (Sigma-Aldrich, St. Louis, MO, USA) at 25 °C.

5.3.2 Lipidomic analysis

The total lipids were extracted following the previously reported method [148] and analyzed by Kansas Lipidomics Research Center Analytical Laboratory (KLRC, Kansas, US) using electrospray ionization (ESI) triple quadrupole mass spectrometry (API 4000, Applied Biosystems, CA, USA) at the Kansas Technology Enterprise Corporation Analytical Laboratory, Kansas State University [149].

5.3.3 Acyl flux analysis with radiolabeled acetic acid and glycerol tracers

A single colony of *Thraustochytrium* sp. 26185 was inoculated into 5 mL of GY medium and incubated at ambient temperature for 24 hours (24 h) before it was sub-cultured into 100 mL of fresh GY medium. When the culture reached early log phase (OD_{600} about 0.5), the cells were harvested by centrifugation at 4,000 rpm at 4°C for 5 min and re-suspended in fresh GY medium to an OD_{600} of 0.5. The culture was then incubated at ambient temperature for 1 h for equilibration before adding tracers for pulse-chase labeling or steady-state labeling.

For pulse-chase labeling by acetate, 10 µCi of [1-^{14}C]-sodium acetate (50.5 µCi/µmol, Perkin Elmer, Waltham, MA, USA) was added to a 10-mL culture. After labeling for 30 min, the *Thraustochytrium* sp. 26185 cells were immediately precipitated by centrifugation and washed twice with 10 mL of distilled water. Afterwards, the cultivation was resumed in 10 mL of GY medium for 3 days. At 30 min of feeding and 3 d post feeding, the total lipids were extracted from the culture samples and used for lipid class, fatty acid composition and positional analysis. For pulse-chase labeling by glycerol, the same procedure was followed except that 100 µCi of [1,3-^{14}C]-glycerol (55 µCi/µmol, American Radiolabeled Chemicals, Inc. St. Louis, MO, USA) was used and the labeling time was 1 h instead of 30 min. No positional analysis was performed for ^{14}C-glycerol labeled lipids.

For steady-state labeling by acetate, 10 µCi of [1-^{14}C]-sodium acetate was added to a 20-mL culture. At the time point of 1, 2, 5, 10, 30 and 60 min, 6 h, 12 h and 24 h after feeding, 1 mL of culture was harvested and used for lipid analysis. For steady-state labeling by glycerol, 50 µCi of [1,3-^{14}C]-glycerol was fed to a 10-mL culture. At the time points of 10, 30, and 60 min, 6 h, 12 h and 24 h of feeding, 1 mL of culture was harvested and used for lipid analysis. Three biological replicates were performed for all labeling experiments.

5.3.4 Lipid analysis

All methods used for lipid analysis were described in detail previously [148] unless stated otherwise. In brief, total lipids were extracted by a modified Bligh & Dyer method [137, 148]. Total lipids were separated into TAG, phospholipids (PL) and DAG on thin layer chromatography (TLC) plates by hexane/diethyl ether/acetic acid (70/30/1, v/v/v). Separated lipids were scraped from TLC plates and extracted from the silica with chloroform/methanol/water (5/5/1, v/v/v) and then followed by phase separation and collection of chloroform. PC was resolved from eluted PL on TLC plates with chloroform/methanol/acetic acid/water (75/25/4/4, v/v/v/v). For fatty acid analysis, fatty acid methyl esters (FAMEs) were prepared from scraped silica gels containing each lipid class by heating at 85 °C for 1 h with 2% sulfuric acid in methanol. FAMEs were then separated into VLCPUFAs and SFAs on TLC plates pretreated with 10% $AgNO_3$ in acetonitrile and developed with hexane/diethyl ether/acetic acid (94/4/2, v/v/v).

Positional analysis of TAG was conducted by a partial digestion using lipase from *Rhizomucor miehei* [150], which de-acylates TAG at the *sn*-1 and *sn*-3 position, producing free fatty acids (FFAs) and monoacylglycerol (MAG). The digestion products were then transmethylated by heating at 85 °C for 1 h with 2% sulfuric acid in methanol and the resulted FAMEs were then separated by $AgNO_3$-TLC. TAG was dissolved in 1 mL of diethyl ether, and 0.8 mL of a buffer containing 50 mM of borate and 5 mM of $CaCl_2$ (pH 7.8) and 200 µL of lipase from *Rhizomucor miehei* (Sigma-Aldrich, L4277, St. Louis, MO, USA) were then added for digestion. The sample was mixed thoroughly by vortexing and incubated at 37 °C for 1 h with shaking at 250 rpm. The reaction was stopped by adding 2 mL of methanol/chloroform (1/1, v/v). The mixture was centrifuged at 2,200 rpm for 10 min and the chloroform phase containing lipids was removed. The mixture was then back extracted with 2 mL of chloroform. The combined chloroform was dried under N_2 gas and the digested lipids were resolved on TLC plates developed with hexane/diethyl ether/acetic acid (70/30/1, v/v/v). The fatty acid composition of resulted FFAs and MAG was analyzed by $AgNO_3$-TLC as described above.

Positional analysis of PC was conducted by digestion of PC using phospholipase A2 (PLA2), following a previously reported method [56] with slight modifications. PLA2 was used to remove fatty acids at the *sn*-2 position of PC, producing free fatty acids and 1-acyl-lysophosphatidylcholine (LPC). PC was dissolved in 1 mL of diethyl ether, and then 0.1 mL of 50 mM Tris-HCl containing 5 mM of $CaCl_2$ (pH 8.7), and 1 µL of PLA2 from porcine pancreas (Sigma-Aldrich, P6534, St.

Louis, MO, USA) were added for digestion. The sample was mixed vigorously at room temperature for 30 min. Afterwards the ether was evaporated under N_2 gas, and 3.8 mL of chloroform/methanol (2/1, v/v) and 1 mL of 0.15 M acetic acid were added. The chloroform phase was collected after centrifugation at 2,200 rpm for 10 min, and then 2.5 mL of chloroform was used to back extract the mixture. The combined chloroform was dried under N_2 gas and the digested lipids were resolved into PC, LPC and FFAs on TLC plates developed with chloroform/methanol/acetic acid/water (50/30/8/4, v/v/v/v). The fatty acid composition of the resulting FFAs and LPC were analyzed by $AgNO_3$-TLC as described above.

5.3.5 Radioactivity measurement

Radioactive bands on TLC plates were revealed by a phosphor-imaging method. The TLC plate as developed above was exposed to a storage phosphor screen (Amersham Biosciences, Bath, UK) for at least 4h. The screen was then visualized by a Typhoon FLA 7000 scanner (GE Health Care Life Sciences, Pittsburgh, PA, USA) equipped with an IP filter. Radioactivity of lipids by ^{14}C-acetate labeling was determined by scintillation counting of FAMEs, which were eluted from $AgNO_3$-TLC with hexane/isopropanol/water (60/40/5, v/v/v) following a previously reported method [148]. Radioactivity of glycerolipids from ^{14}C-glycerol labeling was determined by counting the radioactivity in the aqueous phase after transmethylation and extraction with hexane. Radioactivity as disintegration per minute (DPM) was measured by a liquid scintillation analyzer (Tri-carb 2910 TR, Perkin Elmer, Waltham, MA, USA).

5.4 Results

5.4.1 Lipidomic analysis of *Thraustochytrium* sp. 26185 glycerolipids

Liquid chromatography-mass spectrometry was exploited to profile glycerolipids in one-day and three-day cultures of *Thraustochytrium* sp. 26185, corresponding to the log and stationary phases of growth. As shown in Figure 5-1, *Thraustochytrium* sp. 26185 produced a higher amount of polar lipids, such as PC, than neutral lipids, such as TAG and DAG, in the log phase of growth. At this stage, PC was the most abundant (56.2%) among the glycerolipids while TAG accounted for only 16.0% of the total glycerolipids, followed by LPC (9.6%), and other PLs such as PI, PE, PG and PA. As the culturing time increased, the relative amount of PC decreased, and TAG increased concurrently. At the stationary phase, TAG increased to 47.2% of the total glycerolipids, whereas

PC decreased to 38.7%. This result indicates *Thraustochytrium* sp. 26185is active in synthesizing and pooling two types of glycerolipids, PC and TAG, and the biosynthesis of membrane lipid PC is robust at the log phase, and TAG is largely produced at the stationary stage. Interestingly, the pool of lysophosphatidylcholine (LPC) was also found to be much higher in the log phase than that in stationary phase, implying that the active biosynthesis of PC at the early growth stage might go through the LPC acylation pathway in *Thraustochytrium* sp. 26185 [63].

Next, we looked at distribution of molecular species of two major glycerolipids PC and TAG in *Thraustochytrium* at the two growth phases. As shown in Figure5-2, *Thraustochytrium* sp. 26185 produced a unique fatty acid profile with two groups of fatty acids, VLCPUFAs (22:6n-3, 22:5n-6, 20:5n-3) and long chain saturated fatty acids (SFAs) (15:0, 16:0, 17:0), which made it easy to determine PC and TAG species. All PC species observed at the two stages contained at least one of three VLCPUFAs and no PC species with two SFAs were detected. In contrast, all TAGs at the two stages contained at least one SFA, and no TAG species with three VLCPUFAs were detected. Little difference was observed in the distribution of PC species between the two growth phases except for the increase of odd-chain SFA-containing PC species (37:6, 39:6, 37:5 and 39:5) (number of total carbons: number of total double-bonds) with the concurrent decrease of even-chain SFA-containing PC species (38:6 and 38:5) at the stationary phase, which coincides well with our previous finding that *Thraustochytrium* sp. 26185 could produce a large amount of odd-chain SFAs at a late growth stage [148]. The abundant PC species observed at both stages were those comprising fatty acids equaling 44:12, indicating there were two DHA molecules acylated to these PC species. In contrast, much difference was observed in the distribution of TAG species between the two growth phases. The prevailing TAG species at the log phase were those with three SFAs, while TAG species with one DHA (52/53/54:6) dominated at the stationary phase. TAG species with three SFAs decreased from 63.4% to 35.8%, while TAG species with one VLCPUFA increased from 31.9% to 55.4% and TAG species with two VLCPUFAs increased from 4.7% to 8.8% over the two growth phases. Such shift of TAG species directly reflects the increased flux of VLCPUFAs into TAG at the late stage and an active acyl mobilization between PC and TAG might occur during the two growth stages.

5.4.2 Acyl flux among glycerolipids traced by ^{14}C-acetate

To examine acyl flux among glycerolipids particularly between PC and TAG, radiolabeled ^{14}C-acetate was exploited to trace the incorporation of freshly synthesized fatty acids into these

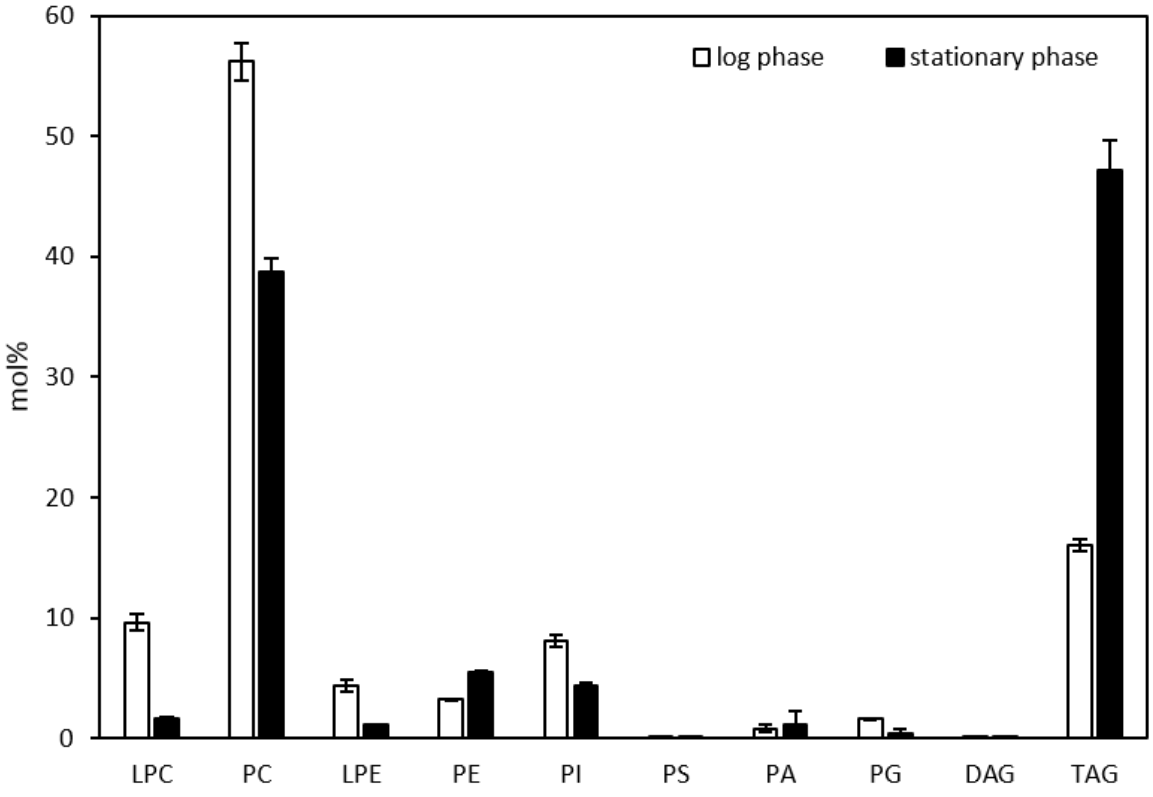

Figure 5-1.10 Glycerolipid composition in *Thraustochytrium* sp. 26185 at the log and stationary growth phases.

The values are molar percentage of total glycerolipids and means ± SD from three biological replicates.

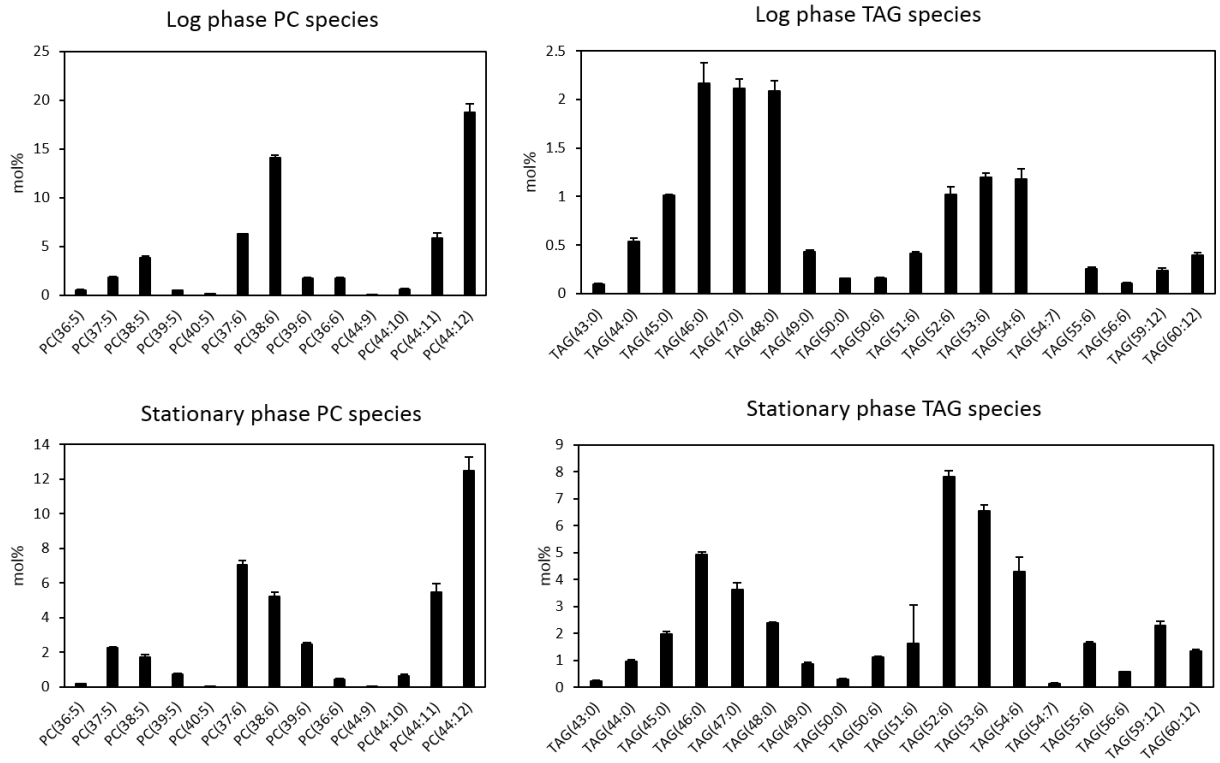

Figure 5-2. Distributions of PC and TAG species of *Thraustochytrium* sp. 26185 at log and stationary growth phases.

The data are presented as the molar percentage of each species in the total glycerolipids. The values are means ± SD from three biological replicates.

glycerolipids using two different protocols, pulse-chase labeling and steady-state labeling. In pulse-chase labeling, *Thraustochytrium* sp. 26185 was fed with ^{14}C-acetate for 30 min. After that, unconsumed radiolabeled precursors were washed away, leaving the cells to grow for another three days (3 d). Given that unlabeled acetate was readily available from the medium, no cold 'chase' was added in this case. At 30 minutes (30 min) of labeling and 3 d post-labeling, the total glycerolipids in *Thraustochytrium* sp. 26185 were analyzed. This protocol was used to examine the ultimate precursor-product relationships of the glycerolipids. In steady-state labeling, *Thraustochytrium* sp. 26185 was fed with ^{14}C-acetate continuously for 24 hours (24 h). The total glycerolipids in *Thraustochytrium* sp. 26185 were analyzed at nine time points (1 m, 2 m, 5 m, 10 m, 3 0m, 60 m, 6 h, 12 h, 24 h) over the time course. This protocol was used to trace the initial incorporation of freshly synthesized fatty acids and acyl trafficking in the glycerolipids.

5.4.2.1 Pulse-chase labeling by ^{14}C-acetate

The total glycerolipids in *Thraustochytrium* sp. 26185 at two time points (30 m and 3 d post-labeling) were extracted and separated into TAG, DAG and PL firstly on a TLC plate, and the PL was then extracted from the plate and further separated on another TLC plate into different phospholipid subclasses, mainly two types of PC, PC1 (PC with one VLCPUFA and one SFA) and PC2 (PC with two VLCPUFAs) (confirmed by positional analysis, see below). As shown in Figure 5-3, the total radioactivity of labeled fatty acids in major glycerolipids including TAG, DAG, PC1 and PC2 remained stable over the two time points. At both time points, most of the labeled radioactivity was found in TAG and PC, together accounting for more than 95% of the total radioactivity, whereas DAG incorporated less than 5% of the total acyl radioactivity. Between the two time points, significant changes in the amount of acyl radioactivity were observed in PC and TAG. At 3 d post-labeling, radioactivity in TAG increased significantly ($P<0.05$) while that in PC decreased significantly ($P<0.05$). Such concurrent changes in the radioactivity of PC and TAG are clearly indicative of an active acyl trafficking between PC and TAG between the two time points. Furthermore, the decrease of radioactivity in PC at 3 d post-labeling was mainly observed on PC1, not on PC2 where the radioactivity at the two time points remained relatively stable, implying that the acyl trafficking might mainly take place between PC1 and TAG between the two time points.
To examine fatty acid profiles of glycerolipids, each lipid class was scraped from TLC plates and transmethylated to fatty acid methyl esters (FAMEs), which were then separated into two groups of fatty acids, VLCPUFAs and SFAs, by $AgNO_3$-TLC. As shown in Figure 5-4, a very different

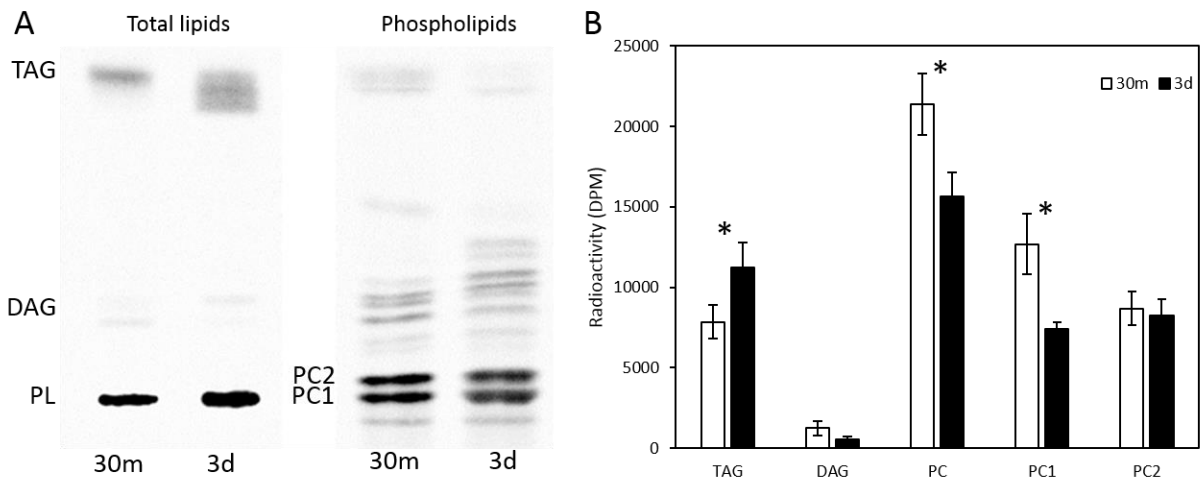

Figure 5-3. Radioactivity distribution in different glycerolipids in pulse-chase labeling by ^{14}C-acetate.

A, TLC separation of total lipids into TAG, DAG, PC1 and PC2; B, Radioactivity in TAG, DAG, PC, PC1 and PC2. The values are means ± SD from three biological replicates. * indicates significant difference ($P<0.05$).

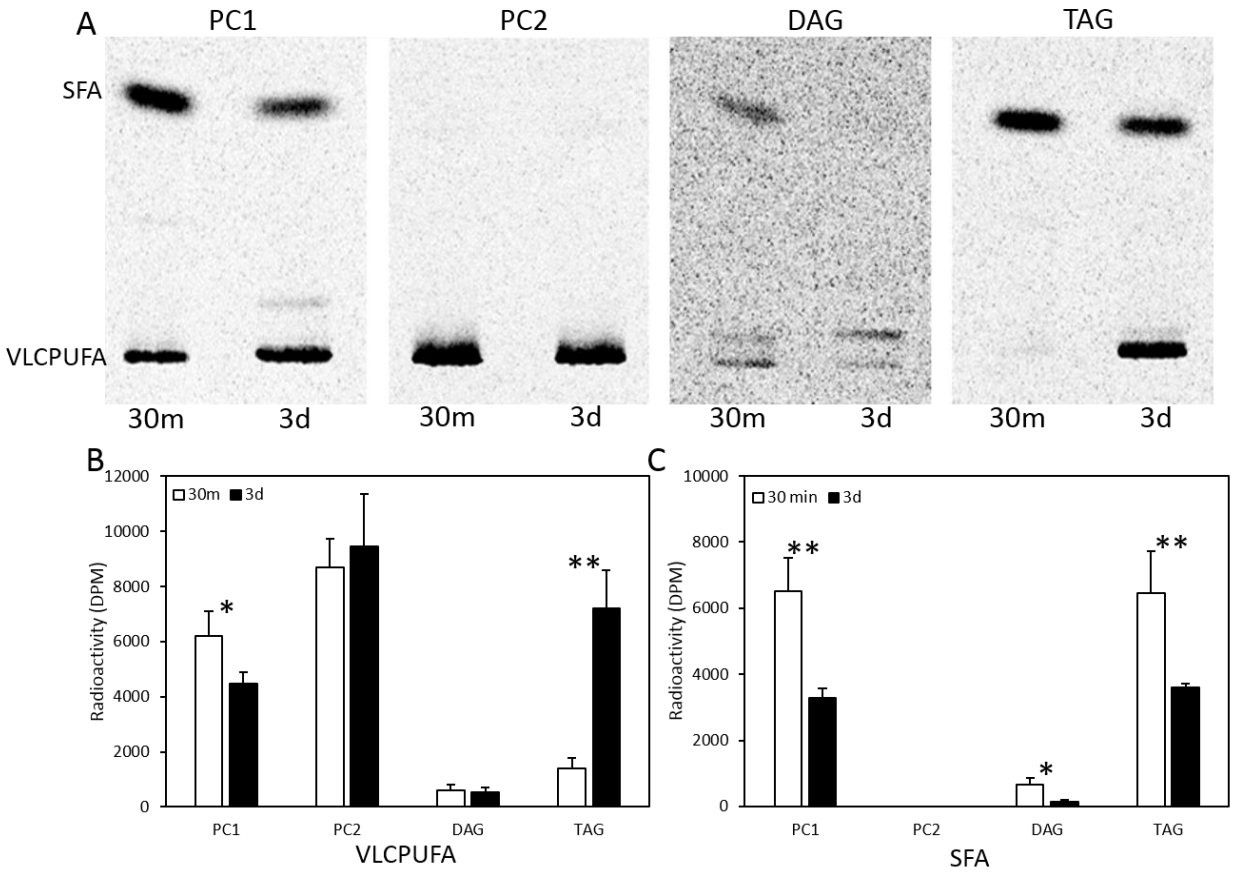

Figure 5-4. Radiolabeled fatty acid profiles of glycerolipids in pulse-chase labeling by ^{14}C-acetate.

A, $AgNO_3$-TLC separation of labeled FAMEs in PC1, PC2, DAG and TAG; B, Radioactivity of VLCPUFAs in the glycerolipids; C, Radioactivity of SFAs in the glycerolipids. The values are means ± SD from three biological replicates. * indicates significant difference ($P<0.05$) and ** indicates significant difference ($P<0.01$).

pattern of ^{14}C labeled fatty acid accumulation in PC, DAG and TAG was observed. PC1 accumulated more ^{14}C labeled SFAs and VLCPUFAs at 30 min of labeling than at 3 d post-labeling. PC2 accumulated exclusively ^{14}C labeled VLCPUFAs and remained relatively stable at both time points. DAG accumulated some ^{14}C labeled SFAs at 30 min of labeling, but very little of them at 3 d post-labeling. On the other hand, TAG accumulated almost exclusively ^{14}C labeled SFAs at 30 min of labeling, whereas at 3 d post-labeling it accumulated more ^{14}C labeled VLCPUFAs than SFAs. The amounts of ^{14}C labeled VLCPUFAs in PC2 and DAG remained relatively constant, while those in PC1 and TAG were significantly different between the two time points. At 3 d post-labeling, the amount of ^{14}C labeled VLCPUFAs in PC1 decreased by 27.7%, while the amount of ^{14}C-labeled VLCPUFAs in TAG increased by about four times. This result clearly indicates the occurrence of VLCPUFA flux from PC1 to TAG.

To investigate possible stereospecific positions of glycerolipids involved in acyl trafficking, positional analysis of PC1, PC2 and TAG from pulse-chase labeling was performed. TAG was partially digested by a lipase, generating two free fatty acids (FFAs) from *sn*-1 and *sn*-3 positions and one 2-acyl-*sn*-glycerol (monoacylglycerol, MAG). PC1 and PC2 were digested by a phospholipase A2, generating one FFA from *sn*-2 position and one 1-acyl-*sn*-glycerol-3-phosphocholine (α-lysophosphatidylcholine, LPC). Afterwards, the fatty acid profiles of digestion products were resolved by $AgNO_3$-TLC. As shown in Figure 5-5, PC1 at both time points comprised one VLCPUFA exclusively-located at the *sn*-2 position and one SFA exclusively-located at the *sn*-1 position. PC2 at both time points comprised two VLCPUFAs with one at the *sn*-1 position and the other at the *sn*-2 position. At 30 min of labeling, ^{14}C labeled TAG comprised almost exclusively ^{14}C labeled SFAs, and all the three positions were occupied by SFAs. However, at 3 d post-labeling, TAG accumulated a substantial amount of ^{14}C labeled VLCPUFAs that were almost exclusively located at the *sn*-2 position, while the SFAs were preferentially located at the *sn*-1/3 positions. This result indicates that the trafficking of VLCPUFAs between PC1 and TAG might mainly occur at the *sn*-2 position of the two.

5.4.2.2 Steady-state labeling by ^{14}C-acetate

To interrogate when and how initial acyl trafficking occurs among glycerolipids, a steady-state labeling experiment of *Thraustochytrium* sp. 26185 was carried out over a period of 24 h using ^{14}C-acetate. The total glycerolipids from the *Thraustochytrium* sp. 26185 cultures at nine time

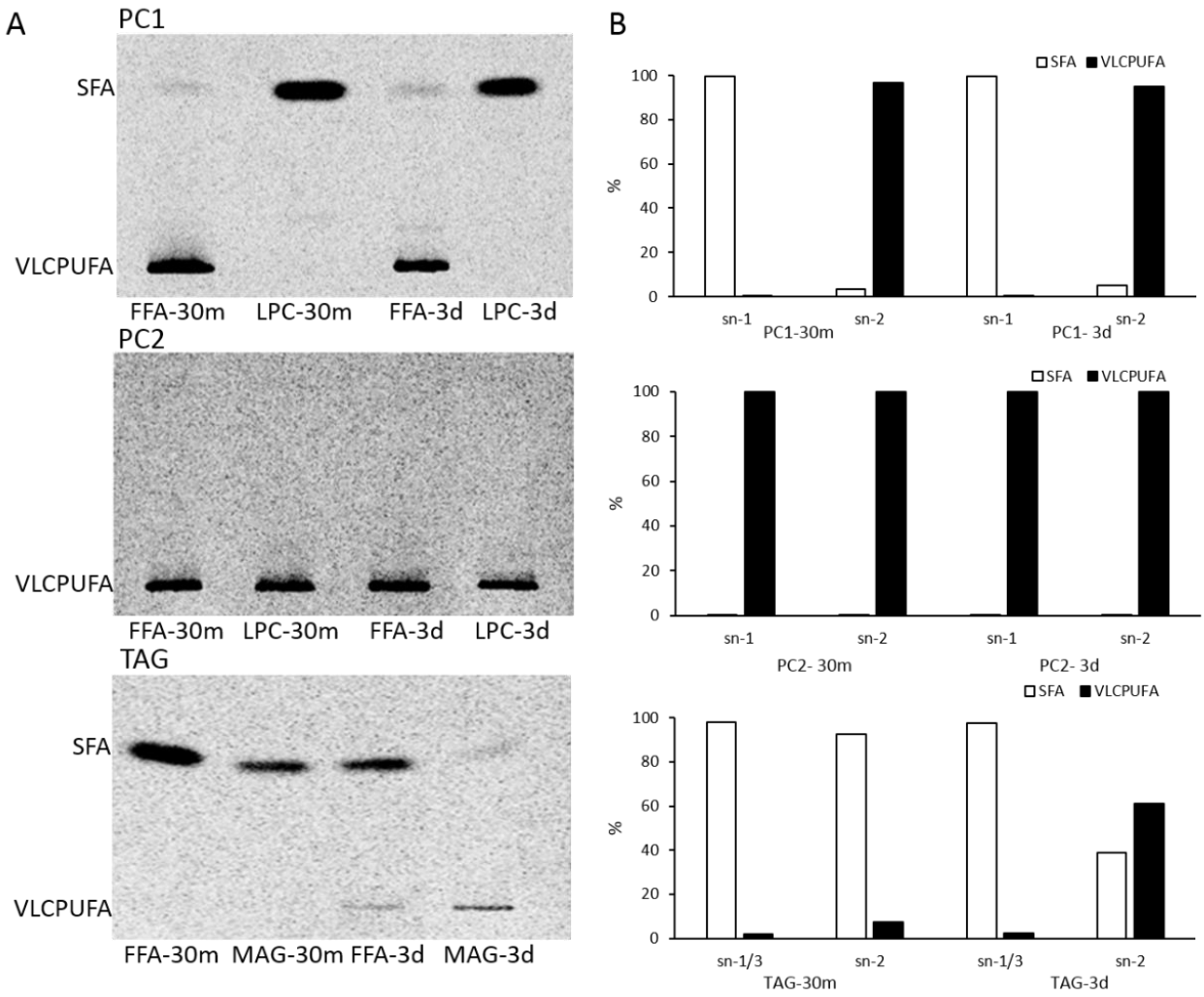

Figure 5-5. Positional analysis of PC1, PC2 and TAG in pulse-chase labeling by ^{14}C-acetate. A, TLC separation of FAMEs of digested PC1, PC2 and TAG products; B, quantitative analysis of fatty acid distribution in PC1, PC2 and TAG.

points (1 m, 2 m, 5 m, 10 m, 30 m, 60 m, 6 h, 12 h and 24 h) were extracted and separated into TAG, DAG and PL. Eluted PL was then separated into PC1, PC2 and other PLs. As shown in Figure 5-6, the total incorporation of radioactivity in glycerolipids occurred at a fast-linear rate over the first one hour and reached the maximum at 12 h of labeling. After that, the total radioactivity in glycerolipids remained relatively constant. Similar to the pulse-chase labeling, most of the labeled fatty acids went into two major glycerolipids PC and TAG with a small amount into DAG throughout the labeling course. The initial incorporation rate in the first minute of labeling was 2.10, 1.27 and 0.01 nmol of ^{14}C-acetate for PC, TAG, and DAG, respectively, with PC being the fastest and DAG being the least in labeling. At 12 h of labeling, PC (PC1 + PC2) accumulated almost two thirds of the total radioactivity in glycerolipids. This result indicates that freshly-synthesized fatty acids were more efficiently incorporated into PC than TAG initially, particularly in the first 6 h. After 12 h of labeling, total acyl radioactivity in PC reached the maximum; afterwards the radioactivity started to decrease. On the other hand, incorporation of labeled fatty acids into TAG was slow over the initial period of 6 h. After that, the incorporation started to pick up and surpassed PC at 24 h of labeling. Such shift of the incorporated radioactivity in PC and TAG reaffirms that acyl trafficking occurs between PC and TAG as observed in the pulse-chase labeling. A further comparison of radioactivity in PC1 and PC2 showed that incorporation of freshly synthesized fatty acids was much faster in PC1 than PC2, particularly over the first 6 h of labeling. After that, radioactivity in PC1 started to decrease. However, radioactivity in PC2 increased continuously after 6 h and reached the maximum at 12 h. Nevertheless, radioactivity in PC1 remained higher than that in PC2 throughout the entire labeling course. Interestingly, while PC1 started to lose radioactivity after 6 h, TAG and PC2 started to pick up the radioactivity. Such a shift of radioactivity accumulation suggests that PC1 is the major donor of acyl groups to TAG and PC2 and the acyl trafficking starts rapidly only after the 6 h of labeling.

To examine fatty acid profiles of glycerolipids, PC, TAG and DAG were scraped from the TLC plate and transmethylated to FAMEs, which were then separated into VLCPUFAs and SFAs by $AgNO_3$-TLC. As shown in Figure 5-7, DAG accumulated a higher amount of ^{14}C labeled VLCPUFAs than ^{14}C labeled SFAs throughout the time course, whereas TAG accumulated a higher amount of ^{14}C labeled SFAs than ^{14}C labeled VLCPUFAs over almost the entire time course except at 24 h of labeling. PC1 incorporated ^{14}C labeled VLCPUFAs and ^{14}C labeled SFAs at a similar rate in the first 6 h, and after that it started to lose both fatty acids. On the other hand, incorporation

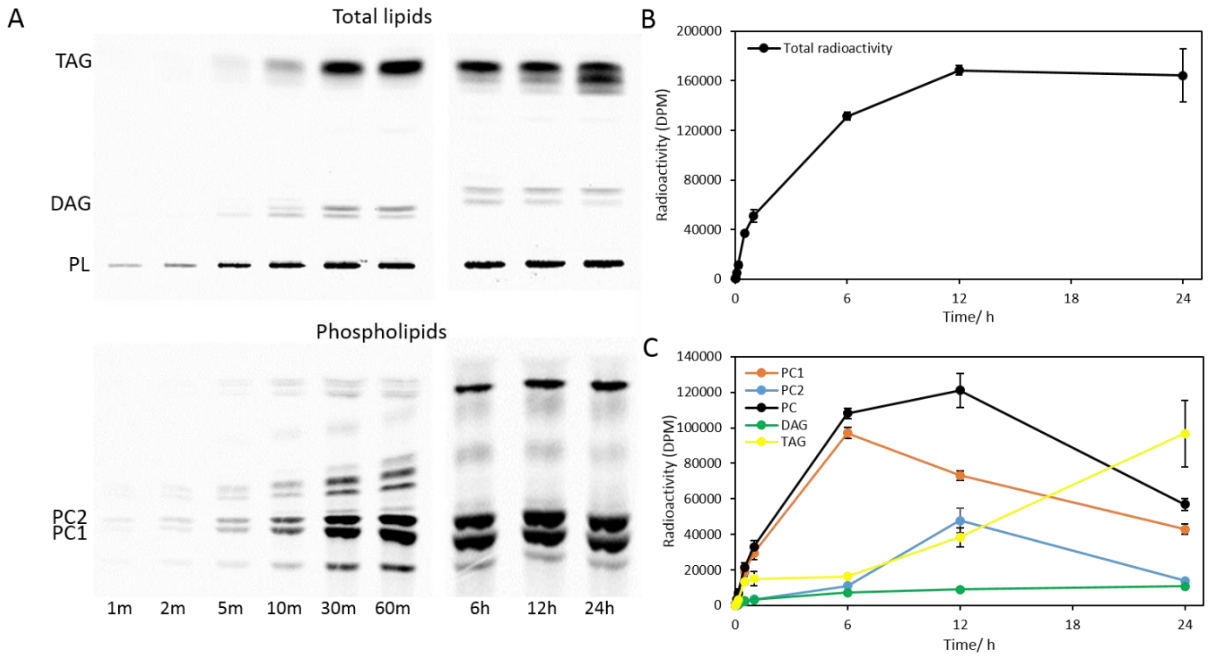

Figure 5-6. Incorporation of ^{14}C-acetate-labeled fatty acids into different glycerolipids in a 24-h time course.

A, TLC separation of total glycerolipids into TAG, DAG and PL, and PL into PC1 and PC2; B, Total radioactivity in glycerolipids; C, Radioactivity in PC1, PC2, total PC, DAG and TAG. The values are means ± SD from three biological replicates.

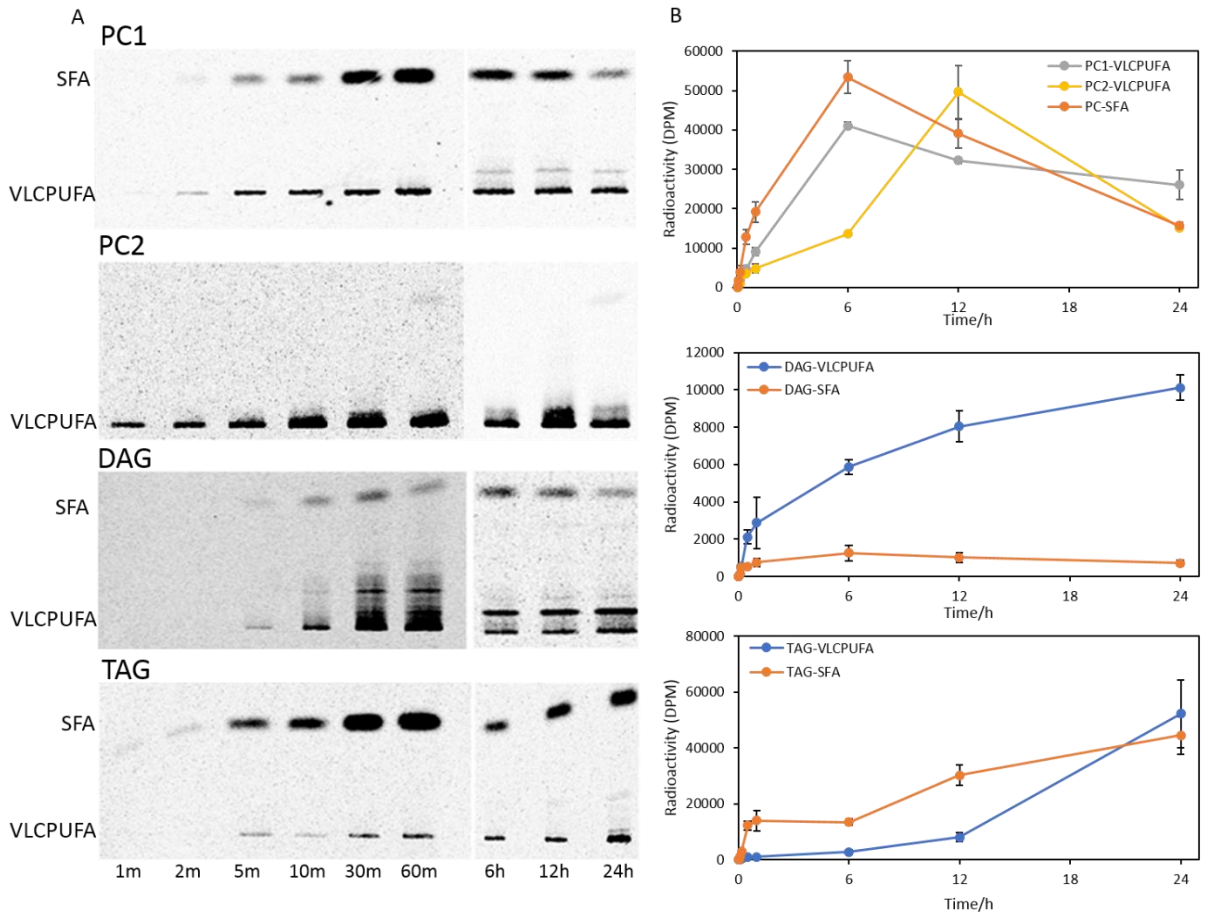

Figure 5-7. Incorporation of ^{14}C-acetic acid-labeled VLCPUFAs and SFAs into different glycerolipid classes.

A, $AgNO_3$-TLC separation of PC1, PC2, DAG and TAG FAMEs; B, Radioactivity of VLCPUFAs and SFAs in PC, DAG and TAG. The values are means ± SD from three biological replicates.

of ^{14}C labeled VLCPUFAs in PC2 was slow in the first 6 h, and after that it started to grow rapidly, and reached the maximum at 12 h of labeling, after which it also started to lose VLCPUFAs dramatically.

A further comparison of fatty acid profiles in different glycerolipids revealed very different patterns of ^{14}C labeled VLCPUFA incorporation (Figure 5-8A). PC entered an almost linear incorporation of ^{14}C labeled VLCPUFAs for the first 6 h, whereas TAG accumulated ^{14}C labeled VLCPUFAs considerably slower at the same period and collected only 7.6% of that in PC at 60 min. DAG collected more ^{14}C labeled VLCPUFAs than TAG at 60 min, even though the total amount of radioactivity in DAG was only 21.7% of that in TAG at the same time point. After 6 h, the incorporation of VLCPUFAs in TAG increased and continued, while at the same time the PC lost VLCPUFAs, until TAG had accumulated the majority of the VLCPUFAs by 24 h. These results also indicate that SFAs were channeled along with VLCPUFAs from PC1 to TAG (Figure 5-8B).

5.4.3 Backbone flux among glycerolipids traced by ^{14}C-glycerol

The above ^{14}C-acetate labeling revealed an acyl trafficking between PC and TAG; however, it remained to be determined what would happen to the glycerol backbone when the acyl groups were transferred. Therefore, similar protocols of pulse-chase labeling and steady-state labeling by ^{14}C-glycerol were also performed to trace the incorporation of the backbone among glycerolipids. When *Thraustochytrium* sp. 26185 was provided with ^{14}C-glycerol, both the backbone and acyl groups in glycerolipids were labeled, as glycerol could serve as a precursor for the synthesis of acetic acid, the precursor for the biosynthesis of fatty acids. Therefore, to trace the flux, only the radiolabeled backbone of glycerolipids was measured.

5.4.3.1 Pulse-chase labeling by ^{14}C-glycerol

As shown in Figure 5-9, labeled glycerol predominately went into PC1, PC2 and TAG, whereas DAG accumulated only a small amount of radioactivity at both 1 h of labeling and 3 d post-labeling. At 1 h of labeling, radiolabeled glycerol was more efficiently incorporated into PC1, and then TAG, PC2 and DAG. At 3 d post-labeling, PC1 and DAG reduced backbone radioactivity while TAG and PC2 gained the radioactivity simultaneously. This result coincides with that of the acyl trafficking, revealing that the backbone is channeled along with fatty acids from PC1 to TAG.

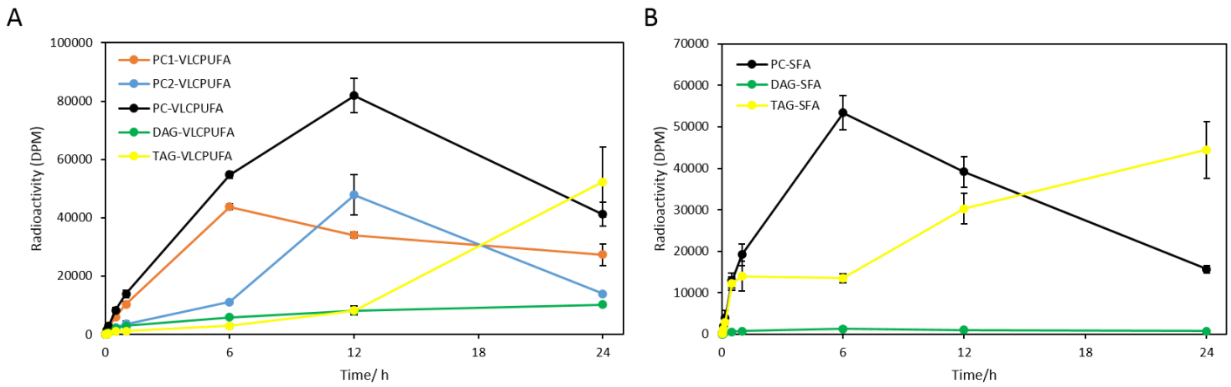

Figure 5-8. Quantitative analysis of ^{14}C-acetic acid-labeled VLCPUFAs and SFAs in different glycerolipids.

A, radioactivity of ^{14}C-VLCPUFAs in PC1, PC2, total PC, DAG and TAG; B, radioactivity of ^{14}C-SFAs in PC, DAG and TAG. The values are means ± SD from three biological replicates.

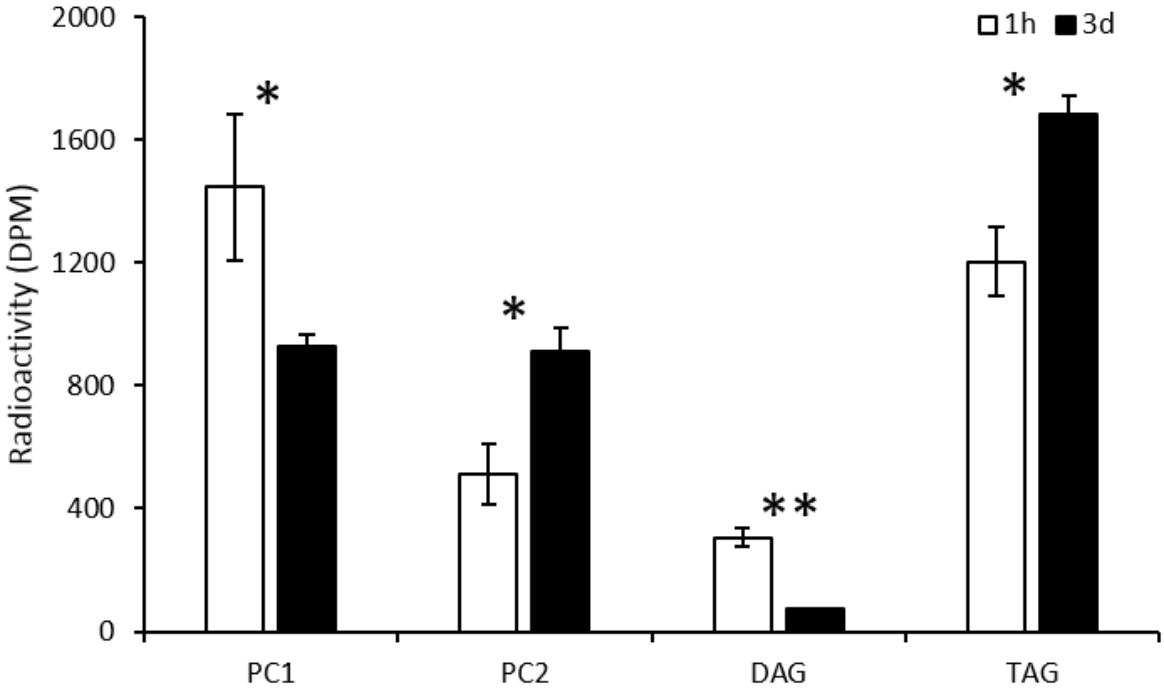

Figure 5-9. Incorporation of ^{14}C-glycerol into PC1, PC2, DAG and TAG as the backbone in pulse-labeling.

The values are means ± SD from three biological replicates. * indicates significant difference of $P<0.05$ and ** indicates significant difference of $P<0.01$.

5.4.3.2 Steady-state labeling by ^{14}C-glycerol

The steady-state backbone labeling of *Thraustochytrium* sp. 26185 was carried out over a period of 24 h. Total glycerolipids in *Thraustochytrium* sp. 26185 at six labeling points (10 m, 30 m, 60 m, 6 h, 12 h and 24 h) in a time course were extracted and separated into TAG, DAG and PL, and eluted PL was further separated into PC1 and PC2. As shown in Figure 5-10, the total backbone incorporation into glycerolipids increased rapidly in the first 6 h and reached the peak at 12 h. The labeled glycerol went predominately into PC (PC1+PC2), and the incorporation in DAG and TAG together accounted only for less than 20% of the total radioactivity at 12 h. Within PC, the incorporation in PC1 was more efficiently than that in PC2 initially. At 6 h of labeling, the radioactivity in PC1 was about 1.4 times of that in PC2. After that, the incorporation in PC1 slowed down, while the incorporation in PC2 speeded up. At 12 h, PC2 incorporated more radioactivity than PC1. On the other hand, TAG collected only 6.5% of the total radioactivity at 6 h, but after that, the incorporation speeded up and overtook all the other glycerolipids at 24 h. Interestingly, DAG displayed a similar incorporation pattern with PC1, despite it incorporated a much less amount of the backbone than PC1. At 6 h DAG accumulated about two times the radioactivity as seen in TAG. After 12 h, it started to lose the radioactivity, and by 24 h the radioactivity in DAG was only about 20% of that in TAG. These results suggest that the backbone trafficking mainly occurs between PC1 and TAG, such trafficking also takes place more vigorously after 6 h of labeling.

5.5 Discussion

In the present study, lipidomic tools and two types of radioactive tracers were used to examine the trafficking process of VLCPUFAs among glycerolipids in *Thraustochytrium* sp. 26185. Lipidomic analysis of the glycerolipids in *Thraustochytrium* sp. 26185 at log and stationary growth stages showed that VLCPUFAs were mostly accumulated in two types of glycerolipids PC and TAG, and an increased mobilization of VLCPUFAs from PC to TAG may have occurred at the latter growth stage. To examine the possible acyl trafficking, ^{14}C-acetate was first exploited to trace the flux of freshly synthesized fatty acids among glycerolipids. When *Thraustochytrium* sp. 26185 was provided with a pulse of ^{14}C-acetate for 30 min, freshly-synthesized VLCPUFAs were initially incorporated into PC rather than TAG, but at 3 d post-labeling, the incorporation in PC was decreased with a concurrent increase of the incorporation in TAG. This result confirms the

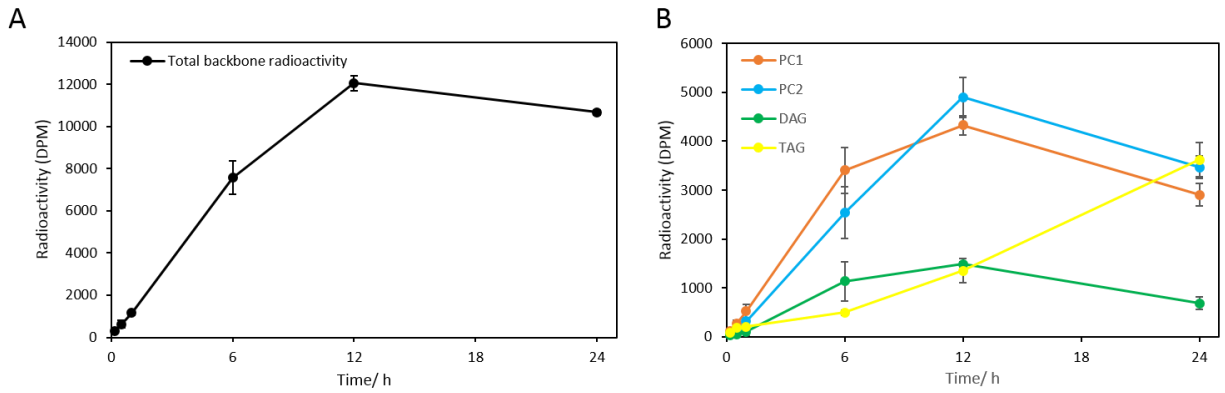

Figure 5-10. The incorporation of ^{14}C-glycerol into different glycerolipid classes as the backbone. A, Total radioactivity in glycerolipids as backbone; B, Backbone radioactivity in PC1, PC2, DAG and TAG. The values are means ± SD from three biological replicates.

occurrence of the VLCPUFA trafficking from PC to TAG. TLC analysis of ^{14}C-acetate-labeled products showed PC in *Thraustochytrium* sp. 26185 was comprised of two types, PC1 (with one SFA at *sn*-1 position and one VLCPUFA at *sn*-2 position) and PC2 (with VLCPUFAs at both *sn*-1 and *sn*-2 positions). It was clear that the decrease of the acyl radioactivity in PC at 3 d post-labeling was mainly observed on PC1, not on PC2, indicating that the acyl trafficking mainly occurs from PC1 to TAG. Furthermore, the decrease of radioactivity in PC1 and the increase of radioactivity in TAG were mainly reflected on VLCPUFAs at the *sn*-2 position of the two glycerolipids, indicating that the VLCPUFA trafficking takes place from the *sn*-2 position of PC1 to the *sn*-2 position of TAG. When *Thraustochytrium* sp. 26185 was provided with a steady supply of ^{14}C-acetate over 24 h, freshly-synthesized VLCPUFAs were more efficiently incorporated into PC1 than TAG where only a very small proportion of total VLCPUFAs were accumulated in the first 6 h of labeling. PC1 started to lose radiolabeled VLCPUFAs while TAG began to gain these fatty acids after 6 h of labeling. This result further indicates that the mobilization of freshly synthesized VLCPUFAs from PC1 to TAG occurs vigorously only after 6 h of labeling.

To investigate the possible involvement of the glycerol backbone in the acyl trafficking between PC and TAG, ^{14}C-glycerol was exploited to trace the backbone flux among the glycerolipids. When *Thraustochytrium* sp. 26185 was provided with a pulse of ^{14}C-glycerol for 1 h, the backbone was more efficiently incorporated into PC1 than TAG, PC2 and DAG. Three days after pulse feeding, the backbone declined in PC1 and mostly incorporated into TAG, indicating an increased backbone flux from PC1 to TAG at the latter stage. When *Thraustochytrium* sp. 26185 was provided with a steady supply of ^{14}C-glycerol for 24 h, PC1 incorporated labeled glycerol much more efficiently than TAG during the first 6 h. After that, the incorporation slowed down. After 12 h, PC1 started to lose the radioactivity. On the other hand, TAG incorporated backbone less efficiently in the first 6 h, but after that, incorporation increased. At 24 h of labeling, the incorporated amount of the labeled backbone in TAG surpassed PC1. This result indicates that the backbone transfer between PC1 and TAG, coincided with acyl trafficking, occurs actively after 6 h of labeling.

Several lines of evidence point to the notion that DAG might be an intermediate transferred from PC1 to TAG. Firstly, the pulse-chase labeling of ^{14}C-acetate and ^{14}C-glycerol showed both SFAs and VLCPUFAs in PC1 were simultaneously-channeled to TAG, coincident with the transfer of backbone that could carry these fatty acids from PC1 to TAG. Secondly, the steady-state labeling of ^{14}C-acetate and ^{14}C-glycerol showed the acyl and backbone trafficking between PC1 and TAG

possessed a similar mobilization trend, actively occurring after 6 h of labeling in the time course. Thirdly, most VLCPUFAs-containing TAG species have similar stereospecific structure as PC1, i.e. SFA at the *sn*-1 position and VLCPUFA at the *sn*-2 position. Fourthly, the steady-state labeling of ^{14}C-glycerol showed that DAG and PC1 shared a similar incorporation pattern, and the decrease of backbone radioactivity in PC1 and DAG are concurrent with the increase of the radioactivity in TAG. Collectively, these results indicate PC-derived DAG is likely an intermediate for the biosynthesis of TAG in *Thraustochytrium* sp. 26185.

There are two distinct pathways for the biosynthesis of TAG using DAG as a substrate in nature. In the acyl-CoA independent pathway, the synthesis of TAG is catalyzed by phospholipid: diacylglycerol acyltransferase (PDAT) transferring an acyl group from the *sn*-2 position of a phospholipid to the *sn*-3 position of DAG [151]. In *Thraustochytrium* sp. 26185, a candidate gene encoding this enzyme was identified in the genome [135]. However, the fact that no VLCPUFA was found in TAG at 30 min of labeling, and only a very small amount of VLCPUFAs were found at the *sn*-1/3 positions of TAG at 3 d post-labeling implies that PDAT might not play a major role in channeling VLCPUFAs to TAG in this protist. In acyl-CoA dependent pathway, biosynthesis of TAG is catalyzed by acyl-CoA: diacylglycerol acyltransferase (DGAT) transferring an acyl group from acyl-CoA to the *sn*-3 position of DAG. Our labeling results indicate that VLCPUFAs channeled to TAG do not come from acyl-CoA pool, but rather from the DAG backbone of PC1 in *Thraustochytrium* sp. 26185. In eukaryotes, DAG is generally believed to be derived from two sequential acylations at *sn*-1 and 2 positions catalyzed by glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT), followed by removing phosphate group at the *sn*-3 position by phosphatidic acid phosphatase (PAP) [152]. DAG derived from PC has rarely been reported in living organisms except for a few plant species where the biosynthesis of highly unsaturated and unusual fatty acids occurs on PC and converting PC to DAG is probably catalyzed by phosphatidylcholine: diacylglycerol choline transferase (PDCT) [91, 93, 119]. In *Thraustochytrium* sp. 26185, candidate gene encoding PDCT was not found in the genome [135]. Therefore, it remains to be determined how DAG is derived from PC in this protist.

In eukaryotes, PC can be synthesized by a few pathways [153]. However, the biosynthesis of VLCPUFA-containing PC in *Thraustochytrium* sp. 26185 might mainly go through an LPC acylation pathway catalyzed by acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT) [154]. Firstly, no genes encoding PDCT catalyzing the exchange of the head group between

existing PC and DAG giving new PC and DAG was found in the genome [135]. Secondly, a small amount of PE present in *Thraustochytrium* sp. 26185 might not be able to support a large capacity of PC synthesis through PE methylation. On the other hand, a high level of LPC, the important intermediate of the LPC acylation pathway was found in *Thraustochytrium* sp. 26185 (Figure 5-2). Thirdly, the ^{14}C-acetate-steady labeling showed that VLCPUFAs were initially incorporated into PC rather than DAG (Figures 5-3 and 5-6), a substrate for PC biosynthesis through a head group activation pathway catalyzed by cholinephosphotransferase (CPT). In *Thraustochytrium* sp. 26185, candidate genes encoding LPCAT and glycerol-3 phosphocholine acyltransferase (GPCAT) were identified, although their functions have not been confirmed [135]. These two enzymes catalyze two sequential acylations of glycerol-3-phosphocholine (G3PC) in the LPC acylation pathway to form PC. In plants, the LPC acylation pathway is commonly used for acyl editing to provide PC-derived DAG with highly unsaturated and unusual fatty acids for the biosynthesis of TAG, as important fatty acid modifications such as desaturation, hydroxylation, epoxidation, and acetylation occur only on PC. However, in *Thraustochytrium* sp. 26185, VLCPUFAs are directly synthesized by a PUFA synthase, and the acyl-desaturation process on PC is deemed unnecessary. Thus, it remains to determine why VLCPUFAs are first incorporated to PC, and then channeled to TAG in *Thraustochytrium* sp. 26185.

In summary, biosynthesis of SFA-TAG in *Thraustochytrium* sp. 26185 at the early growth stage likely uses *de novo* DAG provided by the traditional pathway through two sequential acylations of glycerol-3-phosphate (G3P), while biosynthesis VLCPUFA-TAG at the late growth stage likely utilizes PC-derived DAG provided by an unknown mechanism. Freshly synthesized VLCPUFAs are initially incorporated into PC1, and DAG derived from this PC is a source of VLCPUFAs in TAG. In addition, PC1, to a less extent, can also be a precursor for PC2 probably through an acyl-editing process, and this PC species can also provide DAG for the biosynthesis of TAG (Figure 5-11). Future direction would be to elucidate how PC is converted to DAG, the key intermediate for the biosynthesis of VLCPUFAs-containing TAG in *Thraustochytrium* sp. 26185.

5.6 Acknowledgments

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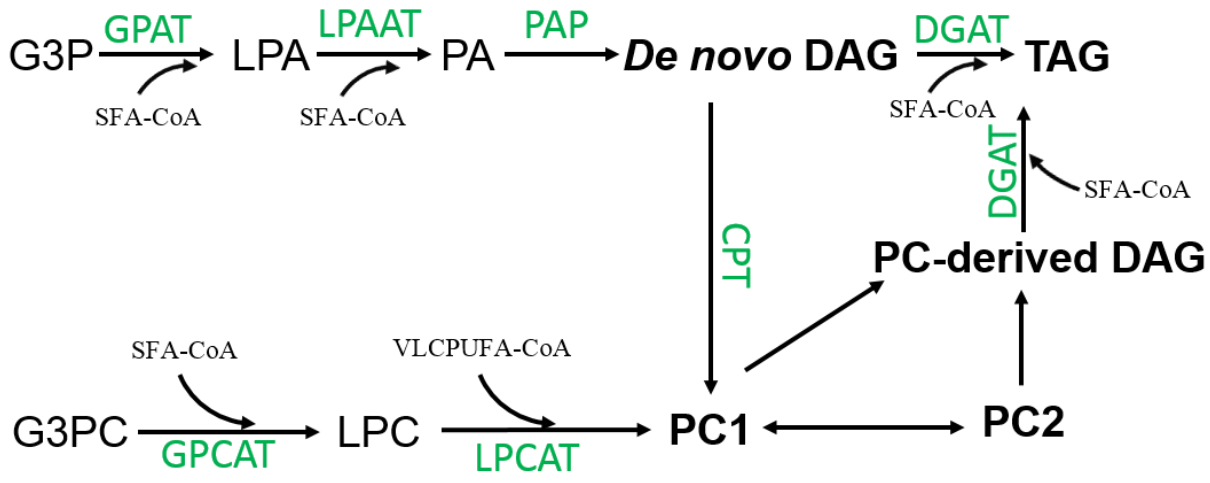

Figure 5-11. Possible pathways of acyl trafficking among glycerolipids in *Thraustochytrium* sp. 26185.

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6. General conclusion, discussion and future directions

There is an increasing demand of VLCPUFAs, such as DHA and EPA, because of their health beneficial potentials. The traditional source of such fatty acids is mainly marine fish oil. However, this source is facing issues such as over-fishing, ocean pollution and oil quality. Extracting oil from VLCPUFA-producing oleaginous microbes from enhanced fermentation or producing VLCPUFAs in transgenic plants by metabolic engineering are attractive alternatives. However, growing VLCPUFA-producing microbes and extracting oil from the microbes are costly, and it is difficult to optimize both biomass accumulation and VLCPUFA production in VLCPUFA-producing microbes. In addition, the level of VLCPUFAs in transgenic plants is still low and undesirable side fatty acids are co-produced in the transgenic oil. The major bottleneck for transgenic production of VLCPUFAs in plants is acyl trafficking from PC to TAG [64]. In transgenic plants with the aerobic pathway for VLCPUFA synthesis, fatty acids are usually desaturated on PC and channeled to acyl-CoA pool for further elongation or to TAG for storage. A better understanding of how VLCPUFAs are synthesized and assembled, and how these processes are regulated in native microbial species, would help to design a strategy to improve the production of these fatty acids in both native and heterologous systems.

Thraustochytrium sp. 26185 is a marine protist that can accumulate a high level of DHA in its storage lipid TAG. It has a very 'clean' fatty acid profile, producing only two types of fatty acids, SFAs such as 16:0 and 17:0, and VLCPUFAs such as DHA and DPA. A previous study on this strain identified several desaturases in the aerobic pathway for VLCPUFA biosynthesis, including the first Δ4 desaturase for the DHA biosynthesis [73]. However, the lack of intermediate long chain PUFAs such as LA and ALA in the aerobic pathway suggests that there might be an alternative pathway, i.e. anaerobic pathway, for DHA production in the species. Thus, the strain is a good model for investigating the relative importance of the two pathways for VLCPUFA biosynthesis. In addition, previous fatty acid profiling revealed that both phospholipids and TAG accumulated a large amount of DHA [72]. However, how DHA is assembled into phospholipids and TAG remains unknown.

In this research, several approaches have been used to investigate the biosynthesis and assembly of DHA in *Thraustochytrium* sp. 26185. Knowledge gained from the research would contribute to our understanding of the biosynthesis and assembly of VLCPUFAs in microorganisms and help improve the production of these fatty acids in transgenic plants. Firstly, we sequenced the entire

genome of *Thraustochytrium* sp. 26185. By sequence annotation, we discovered various genes encoding the desaturases (Δ-4, 5, 6, 12 desaturases and ω-3 desaturase, but no Δ9 desaturase) and elongases (Δ-5, 6, 9 elongases) in the aerobic pathway and three subunits of PUFA synthase, indicating that the protist indeed possesses both aerobic and anaerobic pathways for VLCPUFA biosynthesis. In addition, several pathways in the acyl assembly of glycerolipids such as GPAT, LPAT, PAP, DGAT and CPT were also identified [135]. Secondly, we studied the biosynthetic process of fatty acids in *Thraustochytrium* sp. 26185 using radioisotope-labelled precursors. When fed with radiolabeled intermediate long chain fatty acids such as OA, LA and ALA, no radiolabeled DHA was detected, indicating that the aerobic pathway for DHA synthesis is incomplete. *In vitro* assays using crude proteins showed that VLCPUFAs could be synthesized from malonyl-CoA when the biosynthesis of SFAs by type I fatty acid synthase were inhibited. Feeding with ^{14}C-propionic acid resulted in the production of SFAs but not VLCPUFAs. These results indicate that *Thraustochytrium* sp. 26185 utilizes acetic acid and propionic acid for even-chain and odd-chain SFA synthesis by type I FAS, whereas VLCPUFAs are solely synthesized by a PUFA synthase in *Thraustochytrium* sp. 26185 [148]. Thirdly, we probed the acyl flux of freshly synthesized fatty acids into glycerolipids using radioisotope tracing techniques. In the pulse-chase labeling by ^{14}C-acetate, a strong precursor-product relationship was found between PC and TAG in terms of VLCPUFA accumulation. Steady-state labeling by ^{14}C-acetate revealed that VLCPUFAs were preferentially incorporated into PC initially and only at the later stage of labeling did TAG start to accumulate VLCPUFAs that were channeled from PC. Positional analysis showed that the VLCPUFA trafficking occurred mainly at the *sn*-2 position of PC and TAG. By contrast, SFAs were readily incorporated into both PC and TAG. In the pulse-chase labeling by ^{14}C-glycerol, a strong precursor-product relationship was also found between PC and TAG in terms of backbone accumulation. Steady-state labeling by ^{14}C-glycerol showed a clear backbone trafficking between PC and TAG occurring concurrently with VLCPUFA flux, and a possible intermediate role of DAG in the trafficking. These findings strongly suggest that in *Thraustochytrium* sp. 26185, VLCPUFAs accumulated in storage glycerolipids are channeled from PC, specifically VLCPUFAs are initially incorporated into PC, and PC-derived DAG is used for TAG synthesis. On the other hand, SFAs accumulated in TAG go through the traditional Kennedy pathway.

To the best of our knowledge, this is the first report describing that VLCPUFAs accumulated in TAG are channeled from phospholipids. This finding not only offers insights into the biosynthesis

of glycerolipids in VLCPUFA-producing microorganisms, but also provides strategies for the improved production of VLCPUFAs in microbes and plants by metabolic engineering. As previously mentioned, the bottleneck of producing specialty fatty acids in plants is the acyl trafficking from PC to TAG and oilseed crops do not usually synthesize VLCPUFAs such as DHA and EPA, thus genes/enzymes in the acyl-trafficking pathway from microorganisms can be likely exploited for engineering these fatty acids in oilseed crops.

Accumulation of VLCPUFAs in TAG involves both synthesis and assembly of these fatty acids. The present research has elucidated the mechanisms underlying the biosynthesis and assembly VCLPUFAs in *Thraustochytrium* sp. 26185 using chemical and molecular analysis tools. Nevertheless, there are still questions regarding acyl trafficking among glycerolipids that need to be answered in the protist. Question 1: how are VLCPUFAs actually trafficked from PC to DAG and then to TAG? It is known that PDCT can convert PC to DAG in plants. However, PDCT was not found in *Thraustochytrium* sp. 26185 genome sequences. It is possible that there is a PDCT in *Thraustochytrium* sp. 26185, but it was missed from our genome sequencing or from sequence annotation, as the PDCT in *Thraustochytrium* sp. 26185 might be phylogenetically far away from the known sequences of plant PDCT. Future studies would have to carry out deeper genome sequencing or novel strategies to find the PDCT in *Thraustochytrium* sp. 26185. In addition, it is also known that the reverse reaction of CPT can convert PC to DAG. A candidate CPT gene has been identified from our genome sequencing of *Thraustochytrium* sp. 26185. Future study can aim to generate a *CPT* mutant strain of *Thraustochytrium* sp. 26185 and probe acyl trafficking in the mutant using ^{14}C-acetate and ^{14}C-glycerol tracers. Question 2: How is PC, particularly the most abundant PC species PC1 with a SFA at the *sn*-1 position and a VLCPUFA at the sn-2 position, synthesized in *Thraustochytrium* sp. 26185? PC can be synthesized by several pathways, such as PE methylation and *de novo* choline pathway. However, it is possible that *Thraustochytrium* sp. 26185 utilizes the GPC pathway to synthesize PC. For instance, GPCAT incorporates a SFA at the *sn*-1 position of GPC, producing LPC, which is then converted to PC by LPCAT. To prove this hypothesis, future research can aim to figure out the source of GPC and identify a missing gene encoding GPCAT in *Thraustochytrium* sp. 26185 in the pathway.

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